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A STUDY OF THE EFFECTS OF RENNIN, TRYPSIN, LYSOZYME AND β-GLUCOSIDASE ON CALCIUM CASEINATE, α_s-, β- AND κ-CASEIN

by

© W. JOHN MULLIN

A THESIS
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A Study of the Effects of Rennin, Trypsin, Lysozyme and β-glucosidase on Calcium Caseinate, α_s-, β- and κ-casein

submitted by William John Mullin

in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Chemistry.

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Date March 27, 1974

ABSTRACT

Calcium caseinate, α_S-casein, β-casein and κ-casein were prepared by standard methods and each protein fraction treated with rennin, trypsin, lysozyme and β-glucosidase. In all experiments the proteins were treated with the enzymes at pH 6.3 and incubated for one hour at 30°C. Visual observation of the effects showed that the enzymes induced clotting or precipitating of some of the protein fractions. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed easily recognized monomer bands of the casein fractions and the degradation products of enzyme action. Trypsin had a drastic effect on all fractions, rennin showed a slight effect on calcium caseinate, a marked proteolytic effect on κ-casein and a less severe effect on α_S- and β-caseins. Aggregation of rennin treated α_S-casein was found, also para-κ-casein from rennin treated κ-casein was highly aggregated. The only detected effect of lysozyme was aggregation of α_S-casein. β-glucosidase caused degradation of α_S-casein and slight degradation of β- and κ-casein; aggregation of κ-casein and its products and of calcium caseinate was found.

Analysis of the trichloracetic acid soluble nitrogen from the enzyme treated casein fractions endorsed the gel electrophoresis results. After rennin treatment of κ-casein and β-glucosidase treatment of α_S-casein major amounts of trichloracetic acid soluble nitrogen was found. The bound or associated carbohydrates of the casein fractions were analyzed after hydrolysis and derivatization by gas chromatography. No evidence to support the theory that the carbohydrates were removed by glycolytic enzymes could be found.

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I. INTRODUCTION

The use of milk and milk products as a source of food has been recorded in history as long ago as 2000 B.C. In North America we generally assume the source of milk to be bovine but in other countries the main source may be from a variety of mammals including goats, sheep and yaks; the source being dictated by tradition, climate and terrain. In this dissertation all comments, results and discussion of milk proteins refer to bovine milk. Milk is a rich source of proteins, fats, certain carbohydrates, vitamins, calcium, phosphorus and minerals. Since milk is intended to be the only source of nourishment to the calf for the first few weeks of its life the composition must be adequate to sustain life and maintain growth. Man has taken advantage of this fact and utilized milk as an almost complete source of nourishment. With slight modifications, eg. the reduction of the fat content, cows milk is used as a substitute for human milk in the diet of the young.

Bulk fluid raw milk has the following typical gross analysis for 100 gm (Watt and Merrill, 1963):

Water	87.4	gm	Calcium	118	mg
Protein	3.5	gm	Phosphorus	93	mg
Fat	3.5	gm	Iron	Trace	
Carbohydrate	4.9	gm	Sodium	50	mg
Ash	0.7	gm	Potassium	144	mg
			Vitamin A	140	I.U.
			Thiamine	0.03	mg
			Riboflavin	0.17	mg
			Niacin	0.1	mg
			Ascorbic Acid	1	mg

The composition may vary according to such factors as breed, stage of lactation, frequency of milking, season and climate but in a healthy herd the analysis of a bulk sample should be close to the example quoted above.

Without refrigeration such dairy products as milk, cream, butter, cottage cheese, yogurt and skim milk have a short shelf life. The common food processing techniques, such as drying, freezing, homogenizing, pasteurizing, sterilizing, canning and the utilization of specialized packaging techniques, have been applied to milk and milk products to increase the keeping qualities. Cheese is a highly concentrated milk protein product where certain proteins have been concentrated by an initial 'clotting' process. In cheese the proteins are easily preserved for extended periods and also form a food with a high nutritive value. In all cheese and cottage cheese manufacturing processes, the milk is treated with the enzyme rennin, or a suitable substitute, to form a curd; a bacterial starter culture is added to convert lactose to lactic acid which aids the coagulation process.

In a typical cheese manufacturing process the pasteurized milk, adjusted to the required protein to fat ratio by the addition of skim milk or cream, is put into a stainless steel vat and the temperature raised to about 30°C. After the addition of rennin and/or substitute and the bacterial starter culture, coagulation proceeds. The coagulum is like soft jelly and when firm enough is cut into small cubes using a frame strung with fine wires which is drawn through the coagulum. The whey, which includes the albumins and globulins, lactose, lactic acid and water, drains from the coagulum leaving the coagulated casein proteins behind. The addition of salt to the drained curd acts as a preservative, flavor enhancer and reduces syneresis. The curd is pressed into blocks and then kept in temperature and humidity controlled rooms where aging takes place. The aging process develops the flavor and aroma of the cheese by the continued action of the starter culture, proteolysis due to rennin and enzyme inherent in the milk or by the subsequent action of molds.

The proteins in milk may be divided into two classes, the caseins and the whey proteins. The whey proteins are those which remain in solution after the removal of caseins by precipitation or clotting; the main whey proteins are the albumins and globulins. The casein proteins are in a colloidal complex in the natural state, often referred to as micelles, in association with soluble and colloidal calcium and phosphate. In some of the earliest electrophoretic analyses of the micelle proteins it was found that there were at least three sub classes of proteins in the micelle (Mellander, 1939). These proteins, separated by moving boundary electrophoresis at pH 8.6, were designated α-, β- and γ-caseins in order of decreasing mobility. In fractionating procedures developed later it was established that α-casein was a complex of two separate caseins (von Hippel and Waugh, 1955; Waugh and von Hippel, 1956). These fractions are now designated α_s-casein and κ-casein; α_s-casein being soluble in aqueous solution in the presence of sodium or potassium ions but precipitated in the presence of calcium ions, and κ-casein being soluble in water and not affected by calcium ions. More refined analytical methods and fractionating procedures have revealed that α_s-, β-, γ- and κ-caseins can each be further subdivided into specific genetic types. The very subtle differences in primary and secondary structure of the proteins are breed specific and the analysis of these caseins has now been achieved giving the amino acid composition of the casein fractions, partial sequence of amino acids in α_s-, β-, κ- and γ-caseins, and complete sequence analysis of β-casein A^2 (Ribadeau Dumas et al., 1972).

Molecular weight studies on the individual proteins have given quite a wide range of results (Waugh, 1971). The generally accepted value for α_s-casein is 27,000 daltons, for β-casein 24,000 daltons, both values derived by physical methods. The determination of the molecular weight of κ-casein where the sulphydryl groups have been reduced has given a value of 21,000 daltons. The reported values of the molecular weight of γ-casein show marked variation according to the variant, 22,000 daltons for γ-casein A^3 and 25,000 daltons for γ-casein A^1 (Groves and Townend, 1970). The casein proteins in milk exist in association with each other forming roughly spherical agglomerations or micelles. The micelles are thought to be a sponge like structure in colloidal suspension but many theories exist as to the actual structure itself. The estimated relative abundance by weight of the casein proteins is 40% α_S-, 35% β- and 15% κ-casein; the balance being minor proteins which includes γ-casein. γ-Casein is now considered to be very similar to β-casein with similar primary structure, except that it lacks about 28 amino acid residues from the C-terminal end found in β-casein (Gordon et al., 1972; Groves et al., 1972). The relative amounts of α_S-, β- and κ-caseins has been shown to vary with the size of the micelle (Sullivan et al., 1959; Rose, 1965) but in the proposed models of casein micelles the previously mentioned proportions are used.

In an effort to explain the characteristics of the casein micelle various models have been proposed, five of these are frequently quoted (Morr, 1967; Parry and Carrol, 1969; Rose, 1969; Waugh et al., 1970; and Garnier and Ribadeau Dumas, 1970), and recently another model has been published (Slatterly and Evard, 1973). Taking into account the molecular weights of the component proteins and their relative abundance together with other analytical data; including phosphate and calcium content, effects of proteolytic enzymes, the chemical and physical properties of the component proteins; attempts have been made to propose models which fit the known data. Morr (1967) proposed that small sub units, consisting of aggregates of α_s- and β-casein forming an inner core with an α_s- and κ-casein complex forming the outer layer, were linked together by calcium and colloidal calcium phosphate-citrate bridges between exposed casein carboxyl and phosphoserine groups, to form a roughly spherical micelle. Waugh _et al_. (1970) have proposed that the core polymers are made up of α_s- and β-casein monomers, arranged in a radial configuration, with the more acidic portion of the monomers being nearer the circumference of the rosette type structure. Garnier and Ribadeau Dumas (1970) proposed a model which would account for the permeability of the micelle to high molecular weight reagents and the uniform distribution of the constituent protein monomers. The model envisaged a three-dimensional network with trimers of κ-casein at the nodes and copolymers of α_s- and β-casein branching from them. Rose (1969) has proposed a rather less rigid three-dimensional model which takes into account the predominant association of α_s-casein with κ-casein and includes calcium phosphate into the micelle structure. The β-casein is polymerized end to end and one end of the α_s-casein is attached to the β-casein threads; κ-casein is attached to the free end of the α_s-casein. The structure is randomly coiled with calcium apatite chains linking the proteins stabilizing the structure. Parry and Carroll (1969) have proposed a structure with a κ-casein core and polymers of α_s- and β-casein linked together by calcium phosphate; smaller aggregates were envisaged in the serum having the same κ-casein core but much less α- and β-casein associated with it. This model has been criticized by Fox and Morrissey (1972) who pointed out that the centrifugation methods used did not take into account the dilution factors involved which distorts some of the data used in constructing this model. A model proposed by Slattery and Evard (1973) takes into account many of the factors which are known about the micelle including the higher percentage of κ-casein in small micelles and its predominance on the outer coat. They propose that spherical micelle subunits are made of α_s-, β- and κ-casein with nonuniform distribution of κ-casein giving a hydrophilic area on the surface of the subunit. The rest of the subunit area would be predominantly hydrophobic with calcium binding decreasing the charge on the protein phosphates. Hydrophobic interactions would tend to align the subunits with hydrophilic areas radially outward. Other theories proposed in this model regarding micelle size and the effect of calcium ions further endorses the credibility of this proposal. The estimates of the diameter of the micelle vary considerably but the average, as determined by electron microscopy, has been estimated to be 500-850 angstroms (Carroll et al., 1968); by inelastic light-scattering, using a laser, the average diameter was found to be 800 angstroms (Lin et al., 1971).

Fluid skim milk is a stable colloidal suspension of the constituent proteins and salts; destabilization by such agents as alcohol, acids, heat or proteolytic enzymes reduces the solubility of the proteins. Destabilization includes the terms coagulation, clotting or precipitation and means that the proteins are no longer in a colloidal suspension, the integrity of the micelle has been disrupted and in the case of proteolysis some of the proteins have been hydrolyzed to some extent.

With all the uncertainties of the structure of the micelle it is not surprising that the mechanism of destabilization is not fully understood either. The lowering of the pH to the isoelectric point of the micelle causes destabilization of the comples which can be reversed by raising the pH. Since the discovery of κ-casein its effect on the stability of the micelle has been the subject of intense investigation (Mackinlay and Wake, 1971). α-casein being a complex of α_S- and κ-casein is stable in the presence of calcium. With the addition of calcium to mixtures of purified α_S- and κ-caseins under certain conditions, micelle formation takes place between these two proteins (Noble and Waugh, 1965; Waugh and Noble, 1965). Zittle and Walter (1963) showed that κ-casein stabilizes β-casein against calcium precipitation at 30°C. Even though κ-casein is much less abundant than α_S- or β-casein, the wealth of research indicated that it was the key to the stability of the micelle. Waugh and von Hippel (1956) showed that κ-casein undergoes rapid time dependent precipitation when treated with rennin.

Rennin is a proteolytic enzyme extracted from the fourth stomach of the calf. Berridge (1945) showed that the activity of rennin on denatured hemoglobin was greatest at pH 3.8 and has no activity at all at pH 5.0. However, fresh raw milk has a pH of 6.6 and the clotting action of rennin is fast, dependent upon the reduction of the pH by the action of added starter cultures and the consequent production of lactic acid. The action of rennin on the micelle or on κ-casein increases the amount of soluble organic nitrogen at pH 4.7 (Beeby and Nitschmann, 1963). In the case of κ-casein the precipitate formed after rennin treatment in the presence of calcium ions is called para-κ-casein, the soluble product is called the macropeptide. In 12% trichloracetic acid one-third of the soluble product remains in solution, this portion contains attached carbohydrates and is termed the macroglycopeptide (Nitschmann and Beeby, 1960). κ-casein is the only major micellar protein known to contain a significant amount of bound carbohydrate. The carbohydrate portion is near C-terminal end of the protein, a trisaccharide being attached by an O-glycosidic linkage to a serine residue; the trisaccharide is composed of N-acetylneuraminic acid (terminal), D-galactose and D-galactosamine (Jolles et al., 1962; Jolles et al., 1964; Huang et al., 1964; Baker and Huang, 1967). It is the presence of these carbohydrates, giving a highly hydrophilic character to the C-terminal end of the κ-casein molecule, that is thought to contribute to the stabilizing ability of the protein. It is this protein which is much more susceptible to rennin attack than α_s- or β-casein. The soluble macropeptide and macroglycopeptide, once removed from the micelle, would reduce the hydrophilicity of the complex and thus permit clotting or gel formation in the presence of the bound calcium and phosphate; this theory is included in the models of the micelle. It was not until the publication of work by Delfour et al. (1965), that the specificity of the initial rennin action was clearly demonstrated. Some very elegant analytical work revealed that rennin first attacks a phenylalanine-methionine bond in κ-casein giving rise to the two fragments, para-κ-casein and the macroglycopeptide. Hill (1968) working with synthetic peptides, suggested that the sensitivity of the phenlyalanine-methionine bond to rennin attack was probably due to the catalytic role of a nearby serine residue and to adjacent hydrophobic side chains. As the pH is reduced in the vat during cheese manufacture rennin acts as a non-specific proteolytic enzyme but its initial action is the key to micelle destabilization and curd formation.

The overall milk-clotting reaction can be summarized in the following manner (Garnier, 1963):

$$\text{Micelles} \xrightleftharpoons{Ca^{++}} \text{soluble } \alpha_S\text{-}\kappa\text{-}\beta\text{-casein complex} \tag{1}$$

$$\text{Soluble } \alpha_S\text{-}\kappa\text{-}\beta\text{-casein complex} \xrightarrow{\text{rennin}} \text{soluble } \alpha_S\text{-para-}\kappa\text{-}\beta\text{-casein complex + macroglycopeptide} \tag{2}$$

$$\text{Soluble } \alpha_S\text{-para-}\kappa\text{-}\beta\text{-casein complex} \xrightarrow{Ca^{++}} \text{coagulum} \tag{3}$$

Though relatively little attention has been paid to the action of rennin on α_S- and β-caseins, Ledford et al. (1968) reported the effects of rennet extract on α_S-, β- and whole casein. They found that α_S-casein was attacked more readily than β-casein, the proteolysis of both being much faster in slightly acidic conditions (pH 6.0). The proteolysis of the fractions was also faster than in micellar form indicating the inhibitory effect of the micelle structure to proteolysis. Creamer et al. (1971) showed that β-casein when treated with rennin gave three degradation products, these appeared in succession with respect to time. It was shown that three bonds in β-casein were appreciably more susceptible to proteolysis than others and were located towards the C-terminal end of the protein.

The increase in the world production of cheese has been matched by the increase in yield of milk per cow. With increased yields the number of cows required has been less, also the development of dual purpose breeds has reduced the number of calves available for making commercial rennet. Rennet is the crude rennin extract of calves stomachs used in cheese manufacture.

In rennet the clotting power of the preparation is not entirely due to rennin; another enzyme, pepsin, also derived from the stomach, is responsible for proteolysis of the caseins and thus contributes to the coagulation process. Therefore, to reduce the amount of rennin required preparations of 50% rennet and 50% pepsin (bovine) are commonly used (Davis, 1971). Although some problems are encountered most manufacturers are able to overcome them.

A search for other substitutes has been under continued investigation. Porcine pepsin has been investigated but results were not encouraging (Fox, 1969). Plant proteases, such as bromelin derived from the pineapple, and papain from the tropical melon tree, have been used but produce bitter flavors in cheese. Bacterial rennet substitutes have been studied, proteases from Bacillus subtilis, Bacillus cereus, and Streptococcus faecalis var. liquefaciens were not found to be satisfactory because of bitter flavors formed during the ripening period of the cheese (Babel, 1967). Fungal rennet substitutes have been much more successful in finding acceptance. These preparations have been developed by Japanese researchers from Mucor pusillus. Marketed under the name 'Emporase' by Dairyland Food Laboratories Inc., Wisconsin, and under the name 'Sure Curd' by Chas. Pfizer & Co., Inc., New York, these preparations were approved for use in 1967 in the U.S.A. Approval has not been granted in Canada as yet (C.G. Stinson, 1973) though trials at the University of Alberta have produced acceptable Colby cheese; trials on other types were not so successful.

All the substitutes which have been used are proteases, but it would seem logical that since the carbohydrates in κ-casein are thought to be of prime importance in micelle stability then removal of them should reduce the stabilizing power of this protein. Not all of the macropeptide material liberated by rennin action contains carbohydrates but the portion containing carbohydrates is soluble in 12% trichloracetic acid and therefore should be more hydrophilic. Thompson and Pepper (1962) investigated the effect of neuraminidase, an enzyme specific in cleaving sialic acid, on κ-casein. A total of 69% of the sialic acid was removed from κ-casein after 3 hours of enzyme treatment. This sialic acid poor κ-casein had a significantly reduced stabilizing power towards α_s-casein.

Bakri and Wolfe (1971) reported that lysozyme, an enzyme known to catalyze the hydrolysis of β-1,4-glycosidic bonds between N-acetylmuramic acid and 2-acetamido-2-deoxy-D-glucose, would cause destabilization and clotting of the milk micelle. The clotting activity of lysozyme was compared with the action of rennin, a 10-fold greater molar concentration of lysozyme was required for the same clotting effect as rennin at pH 6.0 in the presence of 0.013 M calcium chloride. Neuraminidase, 0.4166 mg/ml, caused no destabilization in this pH range. It was proposed that lysozyme modified the κ-casein by affecting the carbohydrate moiety, reducing the hydrophilicity and thus causing destabilization of the micelle. It was also found that calcium ions had an inhibiting effect on the action of lysozyme. Wolfe (1971) reported the formation of curd in milk due to the action of lysozyme and β-glucosidase. β-glucosidase being a glycolytic enzyme was also thought to affect the carbohydrate moiety of κ-casein.

Since the novel findings of Bakri and Wolfe (1971) and Wolfe (1971), together with the findings of Thompson and Pepper (1962), gave some indications that the carbohydrate moiety may be removed by glycolytic enzymes, a more thorough study of the nature of the action of β-glucosidase and lysozyme was undertaken. This thesis study was designed to examine the effect these glycolytic enzymes on calcium caseinate and α_s-, β- and κ-casein to gain information with regard to the use of glycolytic enzymes as rennin substitutes. The effect of rennin on these substrates was also examined since it is so widely used in the cheese manufacturing industry and it was felt that the use of some different analytical procedures might lead to more information concerning the action of rennin on the caseins. In the initial work the action of trypsin was studied to illustrate the effects of a non-specific proteolytic enzyme. Descriptions of rennin, trypsin, lysozyme and β-glucosidase and the standard assay methods of these enzymes are outlined in Appendix V.

No novel methods of preparation of casein and casein fractions were used. Although there are many methods available the procedures chosen were common in milk protein research and gave yields which would allow several experiments on each batch. This enabled a comparison of analytical results without having to make adjustments for variations encountered due to slight differences which might occur from batch to batch.

The analytical procedures were primarily designed to test the theory that the trisaccharide in κ-casein would be cleaved by the glycolytic enzymes and also to follow enzyme modification and/or hydrolysis of the proteins.

II. MATERIALS AND METHODS

A. General Considerations

In this project a number of preliminary analytical approaches were used but the work finally centered around three techniques; sodium dodecyl sulphate (SDS) gel electrophoresis of the proteins, trichloracetic acid (TCA) soluble organic nitrogen analysis on the enzyme treated proteins and hydrolysis of the carbohydrates and subsequent analysis of the trimethylsilyl (TMS) derivatives by gas chromatography.

For carbohydrate analysis the enzymes were incubated with the purified proteins and subjected to ultrafiltration. The filtered fractions were hydrolyzed, derivatized and analyzed using gas chromatographic techniques to give both qualitative and quantitative results.

Protein degradation and modification due to enzyme action was analyzed using a relatively new type of gel electrophoresis. This allowed visual assessment of the enzyme treated proteins. Since degradation was found to be evident in a number of cases further analysis of the amounts of nitrogen soluble in 2% and 12% trichloractic acid was carried out.

Zone electrophoresis methods of separating milk proteins have been recently reviewed in depth by McKenzie (1971). Starch and polyacrylamide gels are usually used as the separating medium with the gel concentration and buffer system chosen to facilitate the separation required. To use yet another system where a profusion of methods already existed may have seemed unnecessary, however SDS gel electrophoresis offers some distinct advantages. This modification of polyacrylamide gel electrophoresis was introduced by Shapiro _et al_. (1967) in the study of muscle protein and has subsequently been used by other workers

(Dunker and Rueckert, 1969; McDonagh et al., 1972; Weber and Osborn, 1969). A considerable amount of SDS gel electrophoresis of milk proteins had been carried out before the first published results appeared (Andrews and Cheeseman, 1972; Groves et al., 1972) and the method was invaluable in illustrating the breakdown of the caseins. The proteins were dissolved in SDS, dithiothreitol (DTT) was added to reduce the sulphydryl groups and prevent intermolecular aggregation. The hydrocarbon chain of the SDS is attracted to the hydrophobic residues of the protein effectively coating it, eliminating conformational and charge density differences, giving the molecule an overall negative charge. The proteins migrate to the anode and are separated by a molecular seive effect in the gel, the smaller proteins migrating the farthest. Within limits there is an empirical relationship between the relative distance of migration and the Log_{10} molecular weight. By utilizing the appropriate acrylamide concentration in the gel the molecular weight of the protein can be determined.

Trichloracetic acid can be used to precipitate proteins from solution, Nitschmann and Bohren (1955) showed that after rennin digestion of whole casein there was an increase in the amount of soluble nitrogen in both 2% and 12% TCA. Approximately 1.5% of the total nitrogen became soluble in 12% TCA and 4% in 2% TCA. The amount soluble in 12% TCA was due to the macroglycopeptide which is highly hydrophilic due to the presence of bound carbohydrates. In this study the TCA soluble nitrogen after enzyme treatment of the caseins was used to check the results of the gel electrophoresis work, where clearly defined protein breakdown had been found in some cases. The analysis of the nitrogen was carried out using the normal Kjeldahl digestion procedure and the ammonia measured using an ammonium specific electrode. This electrode gave excellent results and was considerably faster than using the steam distillation and titration method.

Methods for the identification and quantitation of carbohydrates used to be confined to paper chromatographic and colorimetric tests. Analysis by gas chromatographic methods offers great flexibility and sensitivity but it was not until quantitative methods of derivatization were developed that this method became widely used. Methylated or acetylated derivatives were first used and later trimethylsilyl ethers have become the preferred derivatives (Sweeley et al., 1963). The method of preparing these derivatives from methyl glycosides has been thoroughly investigated by Clamp et al. (1967) in relation to glycopeptides and their method was followed in this study.

No expertise in the preparation of relatively large quantities of casein fractions was available in these laboratories at the beginning of this project; no published data concerning SDS gel electrophoresis of milk proteins and very little data on the effects of glycolytic enzymes on the caseins could be found. The exploratory nature of this study required modification and refinement of analytical approaches as initial results became available.

B. Preparation of Casein Fractions

1. Preparation of Acid Precipitated Casein

In the preparation of α_s-, β- and κ-caseins the starting material was acid precipitated casein which was prepared from skim milk. The pH of skim milk was reduced to 4.6 by the slow addition of IN hydrochloric acid with vigorous stirring to prevent areas of very low pH occurring for any length of time. The precipitate was filtered through fine gauze and as much liquid as possible expelled by squeezing the precipitate held in the gauze. The precipitate was dissolved in water to pH 7.0 by the addition of IN sodium hydroxide. The pH was reduced to 4.6, the precipitate recovered, redissolved and precipitated for a third time. This precipitate was kept frozen until required for fractionation.

2. Preparation of α_s-Casein

Crude α_s-casein was prepared by the method of Zittle *et al.* (1959) and purified by alcohol precipitation (Zittle and Custer, 1963). Acid precipitated casein was dissolved in 6.6 M urea then diluted with water to give a urea concentration of 4.6 M. The α_s-casein precipitated out and was recovered by centrifugation. The precipitate was dissolved again in 6.6 M urea containing 0.36 M sodium chloride, the α_s-casein was precipitated by reducing the urea concentration to 4.6 M by the addition of water. The precipitate was recovered, dissolved and reprecipitated as before, washed with water then suspended in water at pH 7.2 by the addition of 1 N sodium hydroxide then dialyzed against six changes of distilled water for 72 hours at 4°C to remove the majority of the urea.

The concentration of the dialyzed solution was determined by the method of Oyama and Eagle (1956) and adjusted to 2% w/v at pH 7.2 then an equal volume of absolute ethanol was added. With the addition of about 0.06 vol of 1 M ammonium acetate in 75% ethanol maximum precipitation was obtained. The precipitate containing the contaminants was centrifuged off and the α_S-casein in the centrifugate was recovered by acidification with 3 N hydrochloric acid to a pH of 5.0. The α_S-casein precipitate was centrifuged off, dissolved in water with the addition of 1 N sodium hydroxide to pH 7.5, dialyzed against six changes of distilled water for 72 hours at 4°C, then freeze dried.

3. Preparation of β-Casein (Aschaffenburg, 1963)

Acid precipitated casein was dissolved in 3.3 M urea at pH 7.5 by the addition of 1 N sodium hydroxide. On reducing the pH to 4.6 with 1 N hydrochloric acid a precipitate formed which was filtered off. The filtrate was adjusted to pH 4.9 with 1 N sodium hydroxide then diluted with water to give a 1 M urea concentration and warmed to 30°C. The precipitate was allowed to settle overnight then collected on a Buchner funnel. The precipitate of crude β-casein was dispersed in 3.3 M urea at pH 7.5 by the addition of 1 N sodium hydroxide. The pH was then reduced to 4.6 and the solution warmed to 37°C. The precipitate formed was removed by filtration, the filtrate was brought to a pH 4.9, then diluted to give a 1 M urea concentration. The precipitate was filtered off, dissolved in water with the addition of 1 N sodium hydroxide, then reprecipitated at pH 4.9. This precipitate of purified β-casein was dispersed in water at pH 7.5 by the addition of 1 N sodium hydroxide, dialyzed for 72 hours against six changes of distilled water at 4°C, then freeze dried.

4. Preparation of κ-Casein (Zittle and Custer, 1963)

Acid precipitated casein was dissolved in 6.6 M urea and acidified to give a pH of 1.3 to 1.4, 1.7 vol of water was added and the mixture allowed to stand for 2 hours. The precipitate which formed was filtered off and the κ-casein in the filtrate precipitated by the addition of ammonium sulphate to give a concentration of 1 M. The precipitate was centrifuged off, suspended in water and dissolved by the addition of 1 N sodium hydroxide to give a final pH of 7.5. The concentration of the κ-casein was determined by the method of Oyama and Eagle (1956) and adjusted to 1% w/v at pH 7.0, then 2.0 vol of absolute ethanol added. 1 M ammonium acetate was added until a thick sticky precipitate was obtained which was filtered off, redissolved and reprecipitated as before. The final precipitate was dissolved in water with the addition of 1 N sodium hydroxide, dialyzed against the six changes of distilled water at 4°C for 72 hours, then freeze dried.

5. Preparation of Calcium Caseinate (von Hippel and Waugh, 1955)

Raw, uncooled milk, from a Holstein cow in the University herd, was brought to the lab immediately after milking. The cream was separated from the milk using a centrifugal separator (McCormick-Deering, Model 2-S, International Harvester Co., Chicago). To 1 litre of skim milk, cooled to 5°C, 60 ml of 2 M calcium chloride solution was added and the mixture centrifuged for 90 minutes at 45,000 x g and 5°C in a Spinco L2-65B Ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif.) using the

#20 rotor. The supernatant was discarded and any residual cream adhering to the walls of the tubes carefully wiped away and the gel removed with a spatula. The gel was resuspended in 0.076 M sodium chloride and 0.133 M calcium chloride using a Waring Blendor. The dispersed gel was recentrifuged for 60 minutes at 90,000 x g at 5°C, #30 rotor, and the precipitate recovered as before. The gel precipitate was dispersed in 0.035 M sodium chloride and centrifuged for 60 minutes at 90,000 x g at 5°C; the precipitate was suspended in 0.035 M sodium chloride again and dialyzed against six changes of distilled water at 4°C for 72 hours then freeze dried.

C. Test for Proteolytic Activity of Lysozyme and β-glucosidase

To test for any proteolytic activity of lysozyme (Calbiochem, San Diego, Calif.) and β-glucosidase (Nutritional Biochemicals Corp., Cleveland, Ohio), due to contamination, the method of Anson (1938) was chosen. Suspensions, 2.5% w/v, of dried hemoglobin powder (Matheson, Coleman and Bell, Cincinatti, Ohio) and solutions of lysozyme and β-glucosidase were made up in buffers ranging from pH 8.2 to pH 4.1. The enzyme solutions were added to the hemoglobin suspensions and incubated for 30 minutes at 30°C, then twice the volume of 0.3 N trichloracetic acid was added. After filtration through Whatman No. 3 filter paper an aliquot of the filtrate was removed and the concentration of soluble peptides determined by the method of Oyama and Eagle (1956), using the Folin-Ciocalteau phenol reagent for color development. Blank determinations were carried out adding the trichloracetic acid before the enzymes.

D. Polyacrylamide Gel Electrophoresis

The method employed was similar to that described by Weber and

Osborn (1969). Polyacrylamide gels, 8.5%, were prepared using 6 vol of 28% w/v acrylamide (Bio Rad Laboratories, Richmond, Calif.), containing 0.735% w/v N,N'-methylenebisacrylamide (Bio Rad Laboratories); 2 vol 0.5 M sodium dihydrogen phosphate buffer at pH 7.1; 1 vol 1% v/v N,N,N',N'-tetramethylenediamine (Baker Chemical Col, Phillipsburg, N.J.) and 10 vol 0.14% w/v ammonium persulphate (Fisher Scientific). The gel tubes measured 100 mm x 6 mm i.d. The anode and cathode buffer solutions contained 0.1% w/v sodium dodecylsulphate (Fisher Scientific) and 0.1 M sodium dihydrogen phosphate buffer at pH 7.1. Samples were prepared for electrophoresis by incubating the proteins in the presence of 1% sodium dodecylsulphate and 0.5 mM dithiothreitol (Calbiochem, Los Angeles, Calif.) at 30°C for 24 hours. Following incubation an equal volume of 60% v/v glycerol-water was added to the dissolved protein to increase the density of the solution, aliquots were applied to the top of the gels with a syringe. Electrophoresis was performed at constant current of 5 mA/tube for 5 hours. The gels were stained in a solution containing 0.2% w/v Coomassie Brilliant Blue (Schwartz-Mann Co., Orangeburg, N.Y.) 30% v/v glacial acetic acid, 20% v/v methanol in distilled water. After staining for 1.5 hours, destaining was carried out in a Canalco Quick Gel Destainer (Canalco Co., Rockville, Ms.), using a destaining solution containing 7.5% v/v acetic acid and 5% v/v methanol in water. After destaining for 25 minutes the gels were placed in a 7% v/v acetic acid solution and left to swell for at least 12 hours. The gels were then stained and destained again and finally stored in 7% v/v acetic acid.

E. Preparation of Trichloracetic Acid Soluble Peptides from Caseins

A suspension of calcium caseinate, 10 mg/ml, was made up in 0.02 M phosphate buffer at pH 6.3. Ten test tubes were placed in a water bath at 30°C and 2 ml portions of the protein suspension placed in them. After the suspensions had reached 30°C, two test tubes were removed, 1 ml of 6% trichloracetic acid (TCA) was added to one tube and 1 ml of 36% TCA added to the other, giving final concentrations of 2% TCA and 12% TCA, respectively. A solution of rennin was made up containing 0.625 mg/ml and 0.08 ml added to each tube. Timing was started with the addition of the enzyme, zero time samples were those already containing TCA. Pairs of test tubes were removed from the water bath at 15, 30, 60 and 120 minute intervals and TCA added immediately as before. The precipitated proteins were spun down using a clinical centrifuge and the supernatant removed and filtered through a Millipore 5 µ filter (Millipore Ltd., Montreal, P.Q.) before nitrogen analysis. This scheme was repeated using 0.12 ml lysozyme solution containing 2.5 mg/ml and 0.12 ml β-glucosidase solution containing 2.5 mg/ml. Blank determinations were carried out using no enzymes at all. The whole scheme was repeated for solutions of α_s- and κ-caseins and for a suspension of β-casein, all protein concentrations were 10 mg/ml.

F. Determination of Organic Nitrogen

1. Kjeldahl Digestion

The digestion procedure described by McKenzie and Murphy (1971) was used; powdered potassium sulphate was placed in a dry 30 ml Kjeldahl flask, 18 M sulphuric acid was added together with mercuric sulphate dissolved in 2 M sulphuric acid. The sample solution was added and a small glass bead to aid refluxing and reduce excessive bumping; the walls of the flask were washed down with a small amount of distilled water.

The flask was transferred to a digestion rack and refluxed vigorously for at least 30 minutes and then allowed to cool.

2. Steam Distillation and Titration

Water was added to the cooled digestion flask to dissolve the potassium sulphate and the contents transferred to a micro Kjeldahl distillation apparatus (Fisher Scientific); a solution of 15 M NaOH and 0.2 M $Na_2S_2O_3 \cdot 5\ H_2O$ was added and steam distillation commenced. The tip of the condenser was immersed in an Erlenmeyer flask containing saturated boric acid solution with methylene blue-methyl red indicator to absorb the distilled ammonia. The contents of the flask was titrated against 0.02 N hydrochloric acid to a grey-lilac end point.

3. Determination of Ammonia Using an Ammonia Electrode

Since a considerable number of determinations were required the use of an ammonia electrode was employed (Orion Research Incorporated, Cambridge, Mass.) which was found to be much faster and at least as accurate as the steam distillation and titration method. After the digestion step the cooled contents of the flask were dissolved in water, transferred to a 250 ml volumetric flask and further diluted. A solution of 10 M sodium hydroxide and 2 M sodium iodide was added which brought the pH into the range of 11 to 13 and formed a complex with mercury. The solution was brought up to volume with water and allowed to cool, all measurements were taken at 25°C. 100 ml of the solution was transferred to a beaker, stirred slowly with a magnetic stirrer and the electrode tip immersed in the solution. The ammonia passes through the hydrophobic gas permeable membrane and dissolves in the internal filling solution of the electrode. When equilibrium had been reached (measurements were taken after two minutes in all cases), a reading was taken on the millivolt range of a Fisher Accumet pH Meter (Expanded Scale Research Model 320). This reading could have been used to calculate the concentration of ammonia but since the samples contained very low levels the "known addition" method was used. This eliminated the time required to prepare standard solutions and obtain a calibration curve, which would have to be carried out daily. In the known addition method 10 ml of standard ammonium chloride solution, of approximately ten times the concentration of ammonia expected in the sample, is added to the solution in the beaker. After equilibrium is reached the new millivolt reading is noted. From the difference in the two readings the concentration of ammonia can be calculated using tables provided by Orion Research Inc.

G. Gas Chromatographic Determination of Carbohydrates

1. Preparation of Trimethylsilyl Carbohydrate Derivatives

The method used for the derivatization was essentially that described by Clamp *et al*. (1967). Samples for analysis were dried in a 25 ml pear shaped flask under reduced pressure using a rotary evaporator. If the samples frothed, due to high protein content, freeze drying was used. The dried samples were kept in a desiccator containing anhydrous calcium chloride overnight. The samples were hydrolyzed using 4 ml of 0.75 N hydrochloric acid in anhydrous methanol, two or three boiling chips were added to prevent bumping and aid cutting any protein adhering to the walls of the flasks. The flasks were placed in an oil bath at 80°C, water cooled condensers were attached and drying tubes, containing anhydrous calcium chloride, fitted to the open end of the condensers. Refluxing continued for 6 hours, the flasks were then removed, cooled and the contents neutralized by the addition of solid silver carbonate. Re-acetylation of amino groups was achieved by the addition of 0.5 ml of acetic anhydride, the mixture was allowed to react overnight. The supernatant was removed using a pasteur pipette, anhydrous methanol was added to the flask, thoroughly mixed with the precipitate and the supernatant removed after the precipitate settled again. These washings were combined with the first supernatant in a 10 ml pear shaped flask, then dried in a rotary evaporator under reduced pressure, and left in dessicator overnight. The dried material was derivatized by the addition of 0.4 ml of pyridine, trimethylsilyl chlorsilane and hexamethylenedisilazane (5:1:1, by volume). Derivatization should have been complete after 30 minutes but samples were allowed to react for at least 1 hour before injection into the gas chromatograph.

2. Gas Chromatography

A Varian Aerograph Model 2100 gas chromatograph (Varian Aerograph, Walnut Creek, California), dual column, fitted with dual flame ionization detectors, was used for all analyses. The U shaped glass columns were 6 feet long, outside diameter 0.25 inches and internal diameter 2 mm packed with Chromosorb W, High Performance, coated with 3% SE-30, 80/100 mesh (Chromatographic Specialties, Brockville, Ontario). The carrier gas flow rate of nitrogen was maintained at 25 ml/min, the air flow rate was 250 ml/min and hydrogen flow rate 30 ml/min. The detectors and injectors were maintained at 250°C, after injection of the sample the column oven temperature was linearly temperature programmed from the initial 80°C to 200°C at a rate of 4°/min. The columns were conditioned at 220°C for at least 12 hours before use. All chromatograms were recorded on a Beckman 10" Recorder, Model 100502 (Beckman Instruments Inc., Fullerton, California) 1 mV span, chart speed 0.5 inches/minute.

H. Ultrafiltration of Proteins

Ultrafiltration was carried out using Millipore Pellicon membrane filtrates (Millipore Ltd., Montreal, P.Q.). The prewetted membrane, 25 mm diameter, was placed on the stainless steel support screen and fitted into the filtration cell (17 ml capacity). The material to be filtered was poured into the cell, the cap screwed tight and then connected to a nitrogen cylinder by copper tubing. The nitrogen cylinder was provided with a two stage reduction valve, a release valve was connected to the copper inlet line to vent the cell after filtration had been completed. The pressure used for filtration was about 50 psi of nitrogen. Three filter membranes were used in this study with nominal molecular weight cut-offs of 100,000, 25,000 and 1,000 daltons. According to the technical data provided with the filters, the 25,000 dalton filter retained 100% of bovine serum albumin in solution (67,000 daltons) and 90% of chymotrypsinogen (25,000 daltons). The 1,000 dalton filter retains 90% of bromocresol green (690 daltons) in solution. No technical data was provided for the 100,000 filter; this filter was intended to remove high molecular weight material and precipitated proteins, it was obvious that this was achieved, giving clear filtrates, but, in some cases, very slow filtration rates.

III. RESULTS

A. Visual Effects

The visual effects of the enzymes on the casein fractions are summarized in Table 1. No effort was made to quantitate these results in any way but they gave a clear indication that in some cases the enzymes were having an effect on the proteins. Where the solutions became turbid the opacity increased but little or no actual precipitation occurred. In the three cases where clotting took place the proteins became insoluble and lumpy and could not be dispersed by shaking. Where precipitate formation is indicated the protein sedimented but could be resuspended by shaking.

B. Proteolytic Activity of Lysozyme and β-glucosidase

Since the gel electrophoresis results gave evidence of protein degradation due to the action of β-glucosidase the two glycolytic enzymes were examined more closely for possible proteolytic activity. The enzymes when electrophoresed individually gave single bands in the gels, SDS gel electrophoresis of ovalbumin after treatment with lysozyme and β-glucosidase showed no new bands appearing in the gels. The results of enzyme treatment of hemoglobin are shown in Table 2. No proteolytic activity could be inferred from these results with respect to lysozyme or β-glucosidase; rennin showed considerable activity at pH 4.1 as expected.

C. Gel Electrophoresis; Calibration for Molecular Weight Calculation

Standards used for calibration are shown in Table 3, chymotrysinogen A was taken as the reference standard. The distance of migration

TABLE 1

VISUAL EFFECTS OF ENZYME TREATMENT ON CASEIN FRACTIONS

Casein Fraction	Untreated	Rennin	Trypsin	Lysozyme	β-glucosidase
Calcium caseinate	turbid	clotted	clear	granular precipitate	granular precipitate
α_S-casein	clear	clear	clear	slightly turbid	clear
β-casein	turbid	turbid	turbid	turbid	turbid
κ-casein	slightly turbid	clotted	clotted	precipitate	precipitate

Calcium caseinate 20 mg/ml; α_S-, β-, κ-casein 10 mg/ml; 0.002 M phosphate buffer at pH 6.3

Rennin and trypsin concentration 0.05 mg/ml

Lysozyme and β-glucosidase concentration 0.30 mg/ml

Incubation 1 hr, 30°C

TABLE 2

OPTICAL DENSITY OF TCA SOLUBLE PEPTIDE SOLUTION AFTER ENZYME TREATMENT OF HEMOGLOBIN (Anson, 1938); COLOR DEVELOPMENT USING FOLIN-CIOCALTEAU PHENOL REAGENT

Treatment	Optical Density				
Lysozyme	0.013	0.018	0.018	0.020	0.015
β-glucosidase	0.004	0.000	0.018	0.006	0.009
Rennin			0.017		0.41
Control	0.005	0.004	0.000	0.000	0.002
pH	8.2[a]	7.0[a]	6.3[a]	5.2[b]	4.1[b]

Average of two determinations

a - 0.05 M potassium phthalate buffer
b - 0.05 M sodium phosphate buffer

was measured from the top of the gel to the centre of the protein band. The calibration graph, Figure 1, shows a good straight line relationship between the Log_{10} molecular weight and the relative mobility. In each run 10 or 12 gel tubes were used including at least one for calibration using a solution containing all six standards. The relative mobilities of each standard varied a maximum of ±0.02 from run to run and usually only ±0.01. The apparent molecular weights of the protein bands in the casein and enzyme treated casein gels were read from this graph. The reported molecular weights in Figures 2-9 are the average of five individual gel runs and the average of two runs in Figures 11-23. These values vary ±3,000 daltons in the 80,000 to 100,000 dalton range and the variation diminishes to ±1,000 daltons in the 15,000 to 45,000 dalton range.

D. Gel Electrophoresis of Caseins after Enzyme Treatment, No Filtration, Figures 2 - 9

1. Preparation of Samples

The proteins--20 mg/ml calcium caseinate, 10/mg/ml--were dissolved or suspended in 0.002 M phosphate buffer at pH 6.3. Enzymes were added, 0.05 mg/ml of rennin and trypsin, 0.30 mg/ml lysozyme and β-glucosidase and incubated for 1 hour at 30°C. 11% SDS and 50 mM DTT were added to give final concentrations of 1% SDS and 0.5 mM DTT, the solutions were incubated for 12 hours at 30°C then an equal volume of 60% v/v glycerol was added. The volume equivalent to the weight of protein indicated on the figure titles was applied to the gels. Other details of the gel electrophoresis were as stated in Materials and Methods (C).

The gels shown in Figures 2, 4, 6 and 8 are of the initial studies carried out in this project. Figures 3, 5, 7 and 9 show the gels of

TABLE 3

STANDARD PROTEINS USED IN SDS GEL ELECTROPHORESIS

Protein	Molecular Weight
[a]Gamma-globulins	160,000
[a]Bovine serum albumin	67,000
[a]Ovalbumin	45,000
[b]Tropomyosin	36,000
[a]Chymotrypsinogen A	25,000
[a]Cytochrome C	12,400

a - Mann Research Laboratories, New York, U.S.A.

b - Gift from Dr. W. McCubbin, Faculty of Medicine, University of Alberta

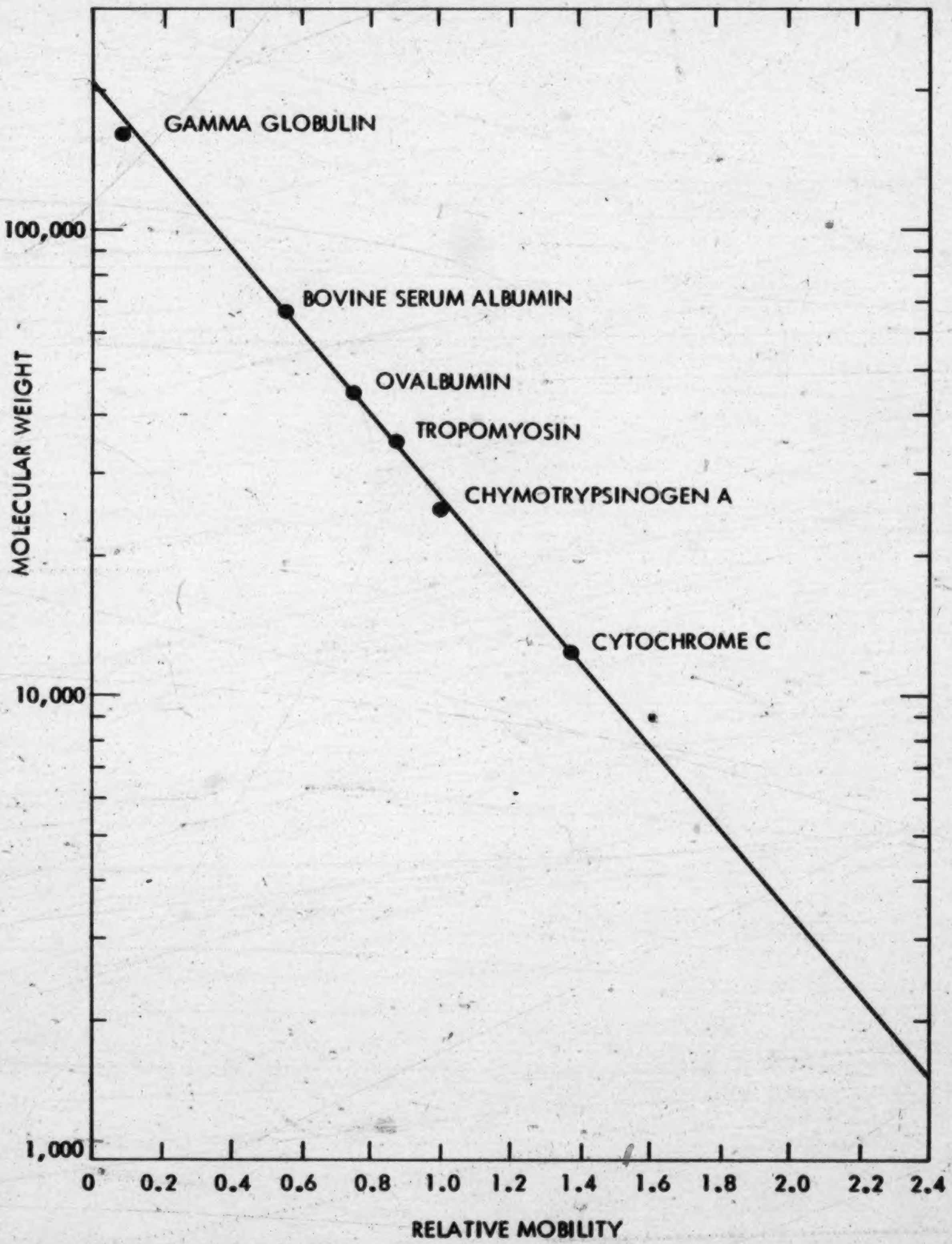

Figure 1. Calibration graph for molecular weight estimations in SDS gel electrophoresis

the proteins after much more pure fractions of the caseins had been obtained; these later preparations were used for all other work reported in this thesis.

Photography and preparation of the gel figures was carried out with all possible care but the final result, in some cases was not as clear as in the gels. Imperfections in the gels and unavoidable incomplete destaining sometimes gave the appearance of bands in the photographs. The following terminology has been used in describing the bands: 'Main band(s)' means the most prominent band; 'minor band' means a band which, compared to the main band, appeared both narrower and less heavily stained; 'faint band' means a band which was discernable on close inspection of the gel and may or may not be discernable in the gel figures.

2. α_S-casein

Figures 2 and 3 show the effects of enzyme treatment on α_S-casein. Untreated α_S-casein (Fig. 2a) showed a main band at 34,000 daltons with minor bands at 91,000; 59,000; 25,000 and 21,000 daltons. The minor bands are due to impurities coprecipitated with α_S-casein. In the rennin treated sample (Fig. 2b) the 25,000 dalton band appeared more intense. Trypsin treatment completely digested the α_S-casein, leaving a band at 9,000 daltons similar to Figure 6c. Lysozyme showed no effect on α_S-casein (Fig. 2c), the band at 12,000 daltons corresponds to lysozyme which was confirmed by electrophoresis of the enzyme alone. Although lysozyme has a molecular weight of approximately 14,000 daltons it is known to migrate abnormally (Dunker and Rueckert, 1969). The other enzymes show no visible bands in the quantities used. β-glucosidase appeared to affect α_S-casein more than the other enzymes (Fig. 2d) causing a considerable increase in 25,000 and 21,000 dalton bands and a decrease in the 34,000 dalton band.

In Figure 3 the effects of the enzyme on the more pure preparation of α_S-casein are shown. The main band is at 34,000 daltons (Fig. 3a) and appears much more discrete than in Figure 2a. The effect of rennin (Fig. 3b) is more marked showing a very clear 25,000 dalton band, and faint bands at 21,000 and 11,000 daltons, β-glucosidase also shows more activity (Fig. 3d) giving a new band at 21,000 daltons and completely removing the 34,000 dalton band; another very faint band is visible at 11,000 daltons.

3. β-casein

In Figures 4 and 5 the gel patterns of untreated and enzyme treated β-casein are shown. The untreated β-casein in Figure 4a compares closely with that of Groves et al. (1972). The molecular weights from the gel pattern of untreated β-casein (Fig. 4a) compare closely with those found by Groves et al. (1972), (27,000; 23,000 and 12,000 daltons for β-casein, γ-casein and TS-, R- and S-proteins, respectively). In Figure 4a the main band at 28,000 daltons was, however, higher than the molecular weight found by other physical and chemical means. The band at 21,000 daltons was probably γ-casein and the band at 10,000 daltons TS-, R- and S-proteins. The rennin treated sample (Fig. 4b) showed a diminished 28,000 dalton band with a band at 22,000 daltons overlapping the 21,000 dalton band. Trypsin completely erased the main bands giving a single band at 9,000 daltons similar to Figure 6c. Lysozyme (Fig. 4c) appeared to have no effect at all, disregarding the 12,000 dalton band. In β-glucosidase treatment (Fig. 4d) a band at 24,000 daltons could be discerned in the gel but not in the photograph, a small band at 22,000 daltons was also visible. In Figure 5,the purer β-casein gel patterns are shown, a very faint band at 10,000 daltons and a main band at 28,000 daltons was visible in Figure 5a indicating the increased purity of this preparation. Evidence of a faint band was visible above the main band, at 32,000 daltons, though it was not completely separated. Rennin treatment (Fig. 5b) had a slight effect on β-casein and a band appeared at 23,000 daltons and faint bands at 11,000 and 10,000 daltons. Lysozyme shows no effect at all on β-casein (Fig. 5c) and β-glucosidase gives a band at 22,000 daltons (Fig. 5d).

4. κ-casein

The results of enzyme treatment of κ-casein are shown in Figures 6 and 7. The untreated protein (Fig. 6a) gave a main band at 26,000 daltons; this is significantly higher than the value of 21,000 daltons for the molecular weight of reduced monomeric κ-casein. The bands of higher molecular weight were probably polymers of κ-casein or aggregates of κ-casein and κ-casein fragments. Rennin treatment (Fig. 6b) substantially reduced the intensity of the 26,000 dalton band and the 65,000 and 57,000 dalton bands; discreet bands became visible at 23,000 and 21,000 daltons. The high intensity band at 12,000 daltons was the para-κ-casein produced by rennin attack but no band corresponding to the molecular weight of the macropeptide or macroglycopeptide (MGP) (8,000 daltons) was visible. The location of the para-κ-casein was confirmed by electrophoresis of a purified preparation. Rennin was added to a solution of κ-casein and incubated, TCA was added to give a final concentration of 12% w/v. The precipitate was removed, homogenized in water, exhaustively dialyzed and freeze dried. The supernatant was exhaustively dialyzed then freeze dried; the two protein fractions were then prepared for application to the gels. The TCA precipitated fraction containing the para-κ-casein gave a band at 12,000 daltons. The TCA soluble fraction gave no visible band after staining, the relatively high proportion of carbohydrates present in the MPP causing abnormal migration and making staining very difficult (Beeby and Mocquot, 1969). Trypsin treatment (Fig. 6c) erased the major bands though a band remained at 12,000 daltons and a new band appeared at 9,000 daltons. No noticeable difference was found with the lysozyme treated sample (Fig. 6d) compared to the control (except for the 12,000 dalton band). β-glucosidase treatment (Fig. 6e) caused some minor changes in band intensity and the 12,000 dalton band increased slightly.

In Figure 7a the κ-casein from later preparations shows the main band at 26,000 daltons; a faint band was visible at 65,000 daltons and 15,000 daltons above a minor band at 12,000 daltons. Rennin treatment (Fig. 7b) showed a large increase in the intensity of the 12,000 dalton band and no evidence of the 23,000 and 21,000 dalton bands. Lysozyme showed very little effect on κ-casein (Fig. 7c), the increased intensity of the 12,000 dalton band is due to lysozyme. β-glucosidase reduced the intensity of the 26,000 dalton band and the intensity of the 12,000 dalton band increased.

5. Calcium Caseinate

The electrophoretic patterns obtained from untreated calcium caseinate are shown in Figures 8a and 8b, the lower loading of the gel in Figure 8a was an attempt to show better separation and definition of the bands compared to Figure 8b. The multiplicity of bands made interpretation difficult. The two lower major bands have apparent molecular weights of 35,000 and 27,000 daltons corresponding to α_S- and β-casein respectively. κ-casein is probably masked by β-casein due to its mobility under these gel conditions and also because of its low proportion in the total micelle complex. The bands at 80,000 and 65,000 daltons are possibly aggregates of casein fractions; those bands of lower molecular weight cannot be identified positively. The gel patterns of rennin, trypsin, lysozyme and β-glucosidase treated calcium caseinate are shown in Figures 8c, 8d, 8e and 8f, respectively. In the rennin treated sample there was a slight increase in the 12,000 dalton band. Trypsin destroyed all bands with a new band visible at 9,000 daltons. Lysozyme had no visible effect on the calcium caseinate but β-glucosidase sharply reduced the intensity of the 80,000 dalton band, a new band at 19,000 daltons was noted and an increase in the intensity of the 11,000 and 12,000 dalton bands.

In Figure 9 the gel patterns of calcium caseinate are shown. The gels showed much better definition of bands though photographic reproduction of these results was found difficult. The preparation of calcium caseinate is much more straight forward than the preparation of the α_s-, β- and κ-casein fractions and there is less chance of the calcium caseinate varying from preparation to preparation. The 21,000 dalton band in Figure 9a is more intense than in Figures 8a and 8b. Other features of the gels in Figure 9 are similar to the corresponding gels in Figure 8, rennin treatment showing some slight increase in the 16,000; 12,000 and 11,000 dalton bands, lysozyme showing little effect and β-glucosidase giving rather blurred bands with increases in the intensities of the lower molecular weight bands.

Figure 2. SDS electrophoreograms of 60 µg of α_s-casein. a, control; b, treated with rennin; c, treated with lysozyme; d, treated with β-glucosidase. Anode is at the bottom.

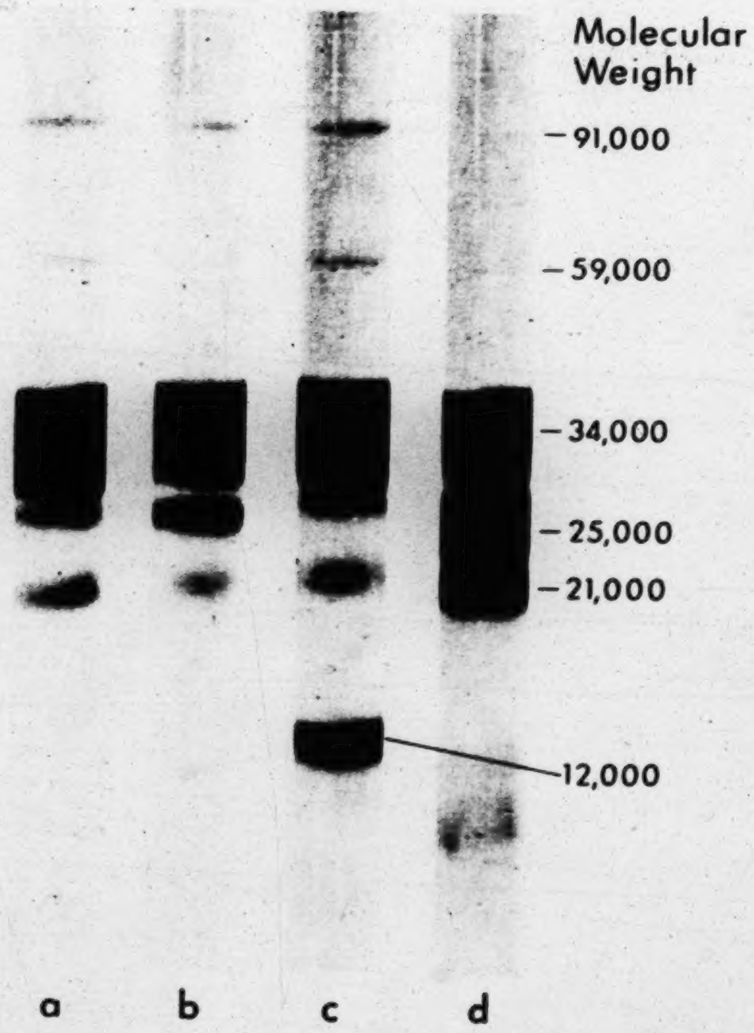

Figure 2

Figure 3. SDS electrophoreograms of 50 µg of α_s-casein (purer preparation). a, control; b, treated with rennin; c, treated with lysozyme; d, treated with β-glucosidase. Anode is at the bottom.

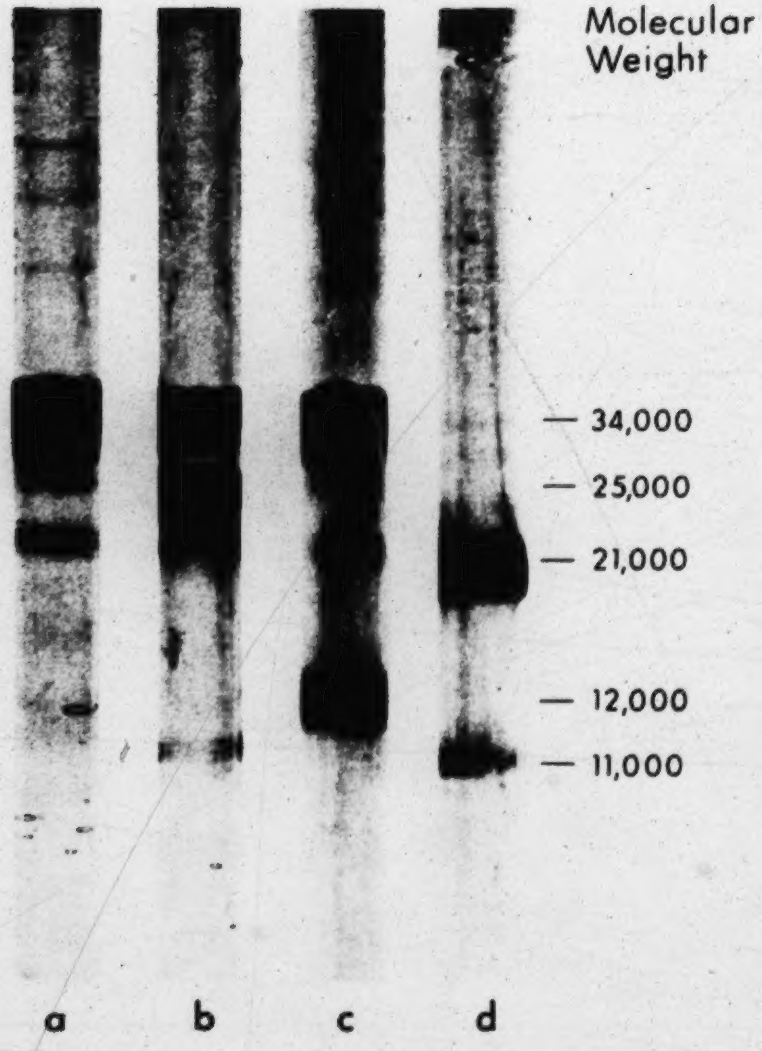

Figure 3

Figure 4. SDS electrophoreograms of 60 μg of β-casein. a, control; b, treated with rennin; c, treated with lysozyme; d, treated with β-glucosidase. Anode is at the bottom.

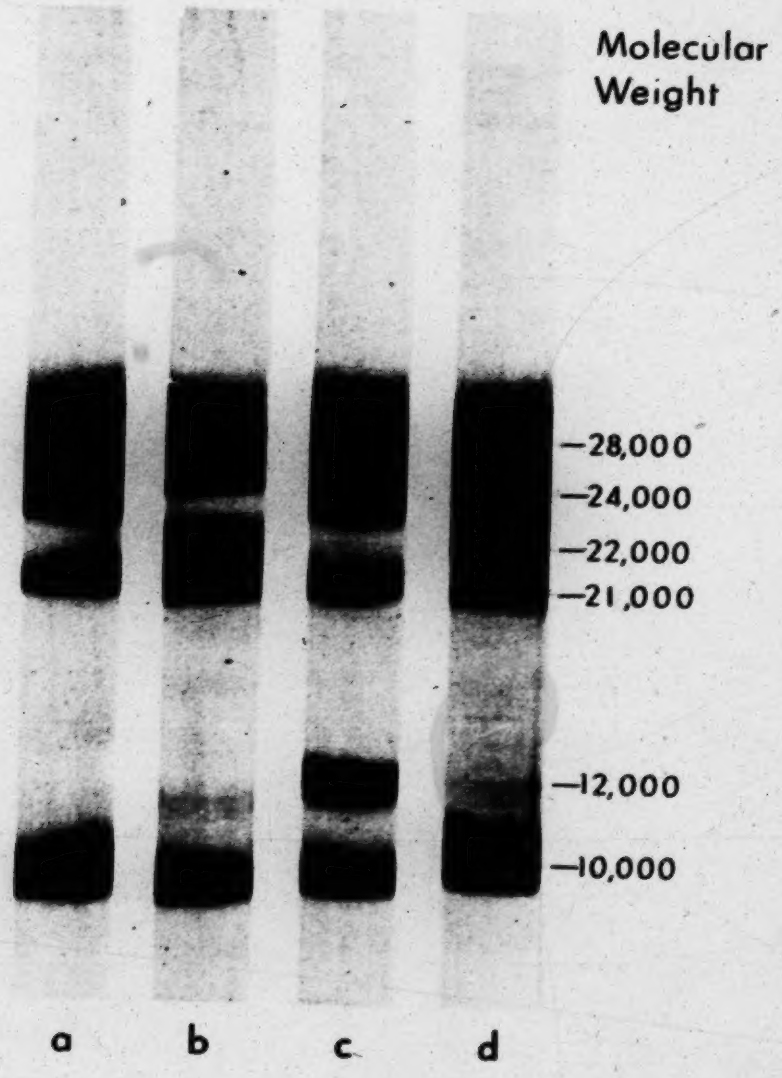

Figure 4

Figure 5. SDS electrophoreograms of 50 μg of β-casein (purer preparation). a, control; b, treated with rennin; c, treated with lysozyme; d, treated with β-glucosidase. Anode is at the bottom.

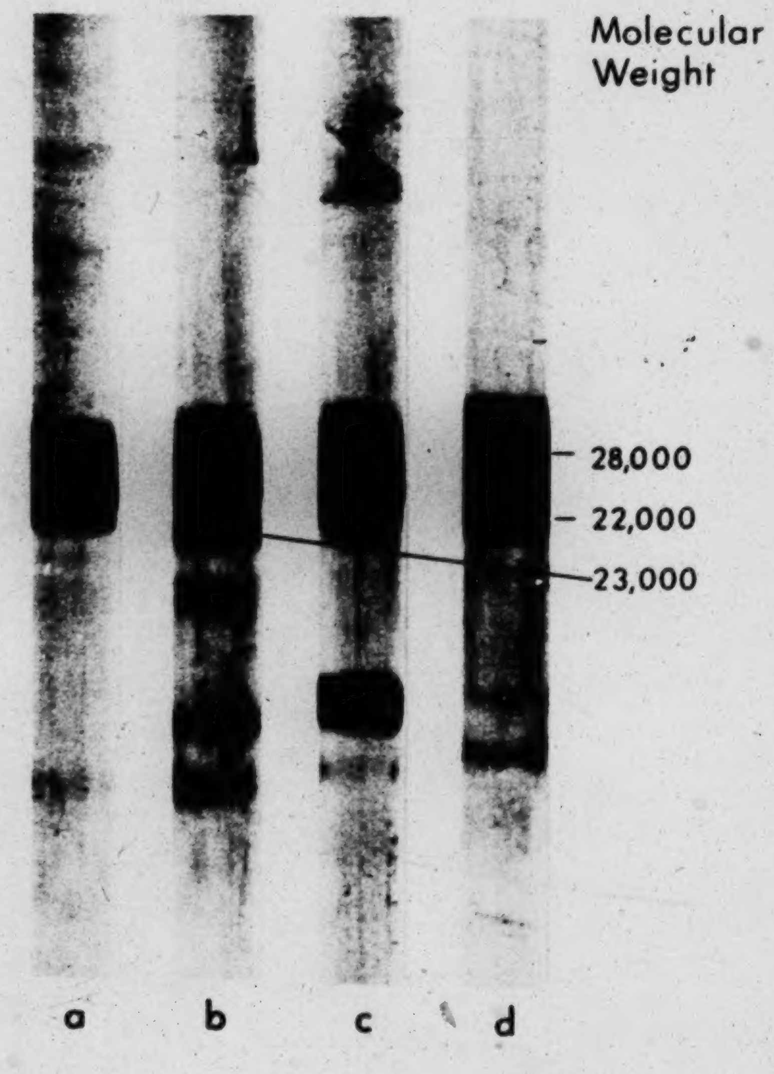

Figure 5

Figure 6. SDS electrophoreograms of 60 μg of κ-casein. a, control; b, treated with rennin; c, treated with trypsin; d, treated with lysozyme; e, treated with β-glucosidase. Anode is at the bottom.

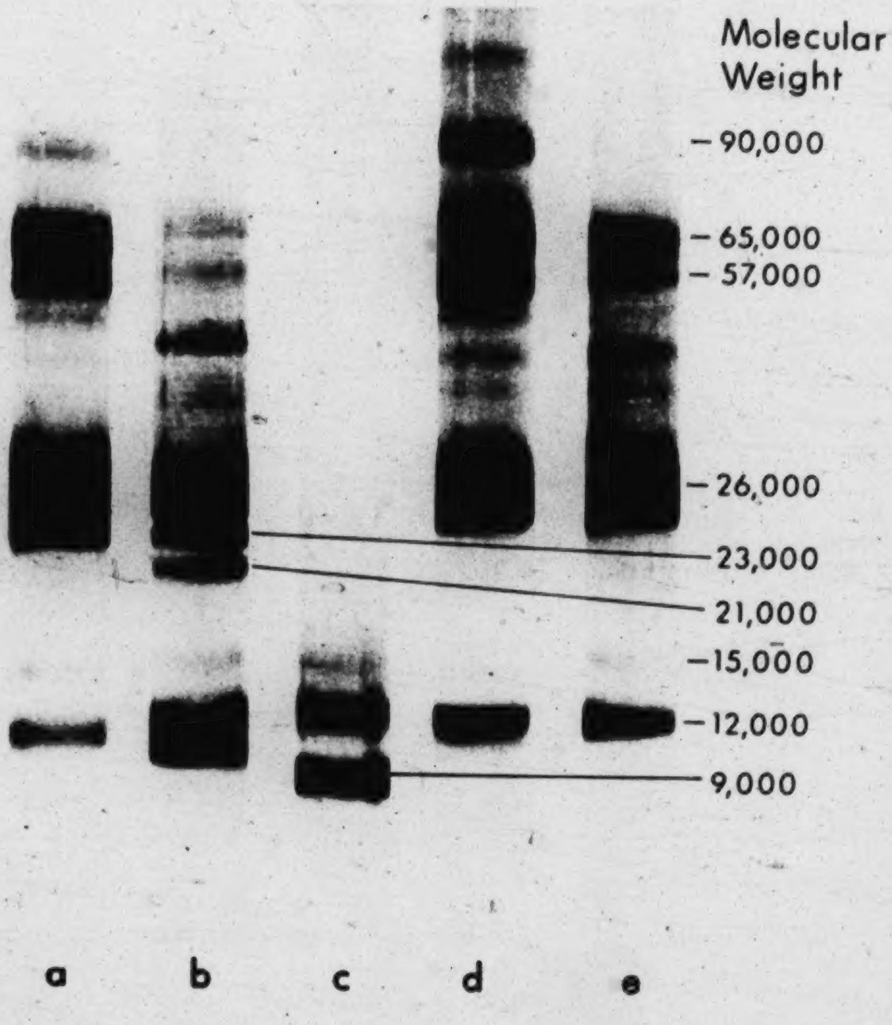
Molecular Weight
90,000
65,000
57,000
26,000
23,000
21,000
15,000
12,000
9,000
a
b
c
d
e

Figure 6

Figure 7. SDS electrophoreograms of 50 μg of κ-casein (purer preparation). a, control; b, treated with rennin; c, treated with lysozyme; d, treated with β-glucosidase. Anode is at the bottom.

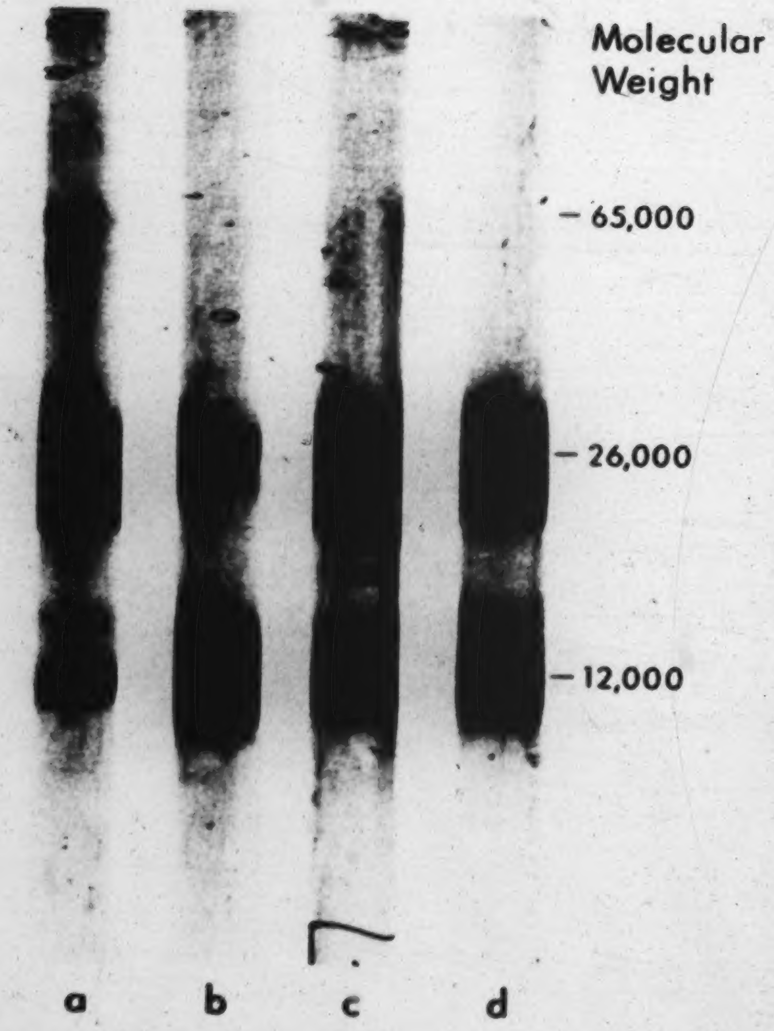

Figure 7

Figure 8. SDS electrophoreograms of 80 μg of calcium caseinate (except a, 40 μg). a, control; b, control; c, treated with rennin; d, treated with trypsin; e, treated with lysozyme; f, treated with β-glucosidase. Anode is at the bottom.

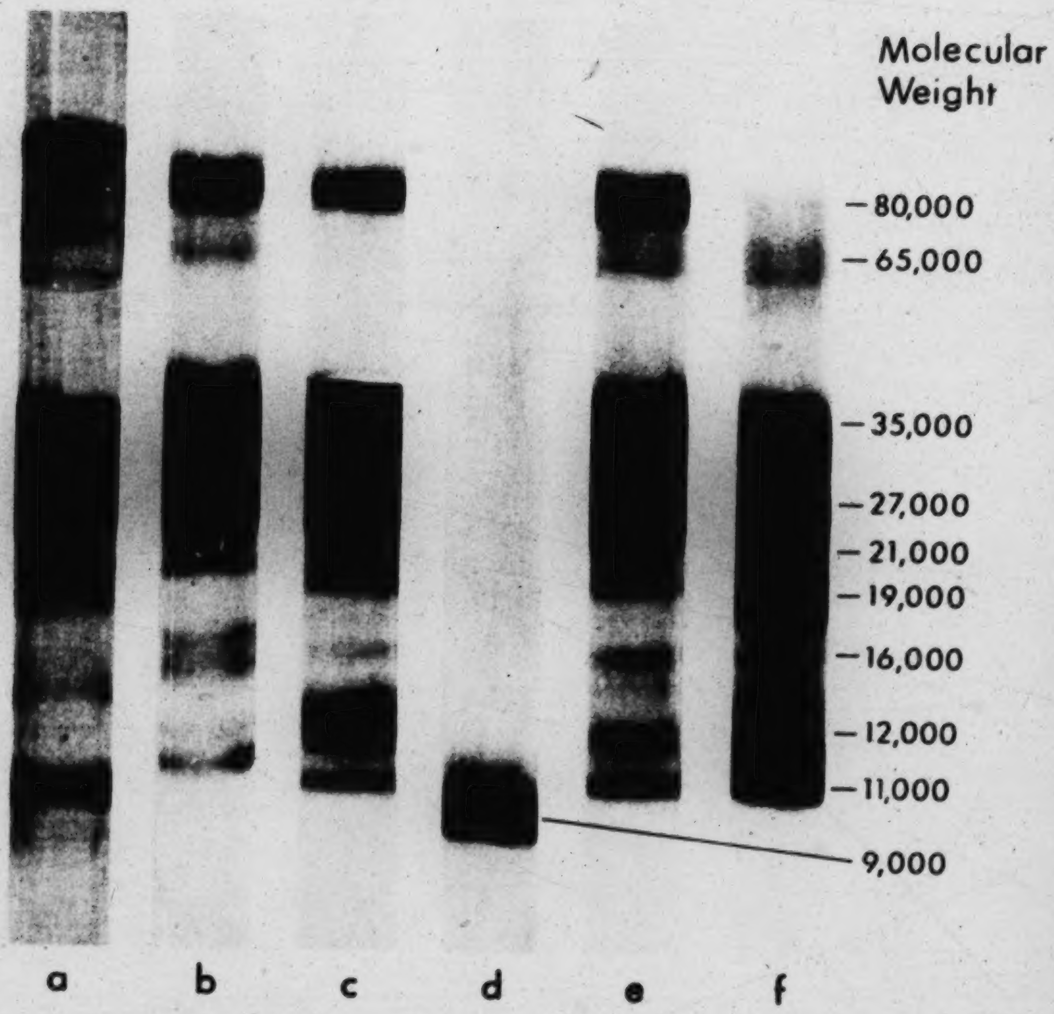
Molecular Weight
80,000
65,000
35,000
27,000
21,000
19,000
16,000
12,000
11,000
9,000
a
b
c
d
e
f

Figure 8

Figure 9. SDS electrophoreograms of 60 μg calcium caseinate. a, control; b, treated with rennin; c, treated with lysozyme; d, treated with β-glucosidase. Anode is at the bottom.

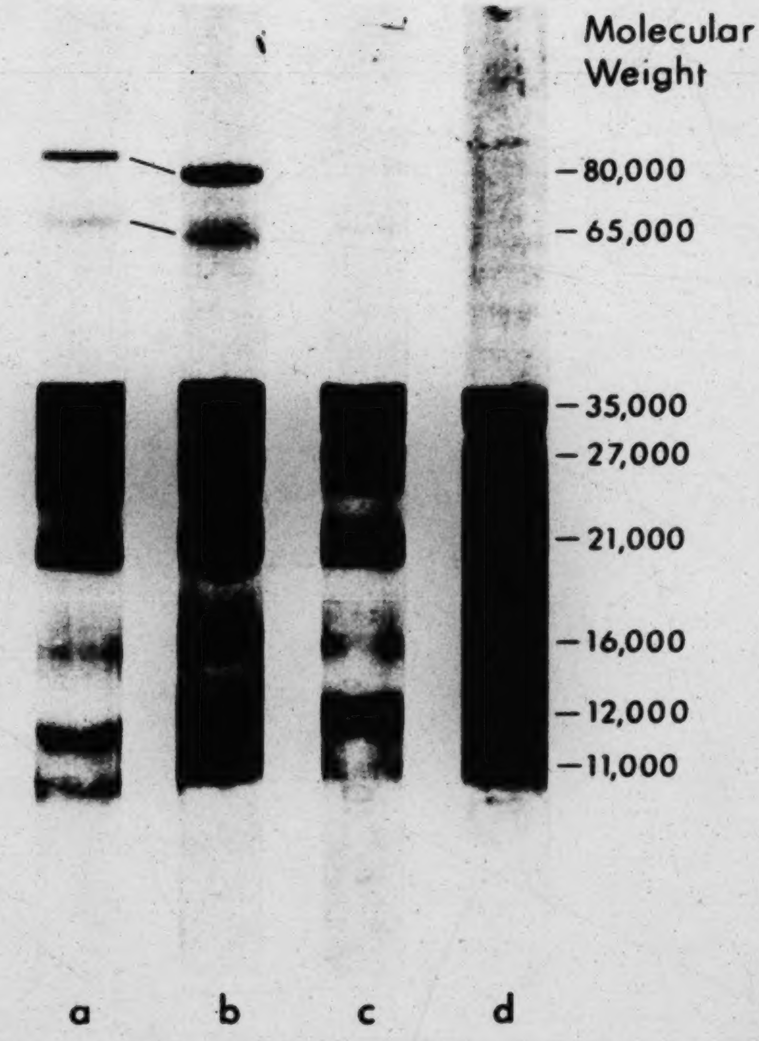
Molecular Weight
-80,000
-65,000
-35,000
-27,000
-21,000
-16,000
-12,000
-11,000
a
b
c
d

Figure 9

E. Gel Electrophoresis of Filtered Fractions

Since a filtration scheme had been planned to eliminate problems in carbohydrate analyses, parallel gel electrophoresis of the fractions was undertaken. A flow sheet of these fractions is shown in Figure 10. The proteins were prepared for gel electrophoresis in 1% SDS, 0.5 mM DTT and 60% glycerol as before. The amount applied to the gels was calculated on the assumption that all the protein would be precipitated after centrifugation, and that no protein would be held back by the filters. On this basis a volume equivalent to 60 μg of protein was applied to each gel. The amount of supernatant solution applied to the gels was calculated in the same way, it was assumed all protein remained in the supernatant. The comparison of the gels would thus give a visual comparison of the protein concentrations in solution and precipitate, and also the amount held back by the filters. No definite quantitative estimates were attempted. It should also be noted that the precipitates were dissolved in 1% SDS before filtration and DTT added afterwards; the supernatants were filtered in aqueous solution then SDS and DTT added afterwards, therefore the supernatants would be more aggregated. Photographs of gels where no protein bands were visible have been omitted.

1. α_S-casein

The untreated α_S-casein gels are shown in Figures 11aS, 11bS1 and 11cS2. The only band visible had an apparent molecular weight of 34,000 daltons. On filtration this band showed reduced intensity intimating that the α_S-casein monomer was polymerized to some extent in solution (Fig. 11bS1, 11cS2). No precipitate was formed with rennin treatment of α_S-casein. The unfiltered sample, Figure 11dS, showed

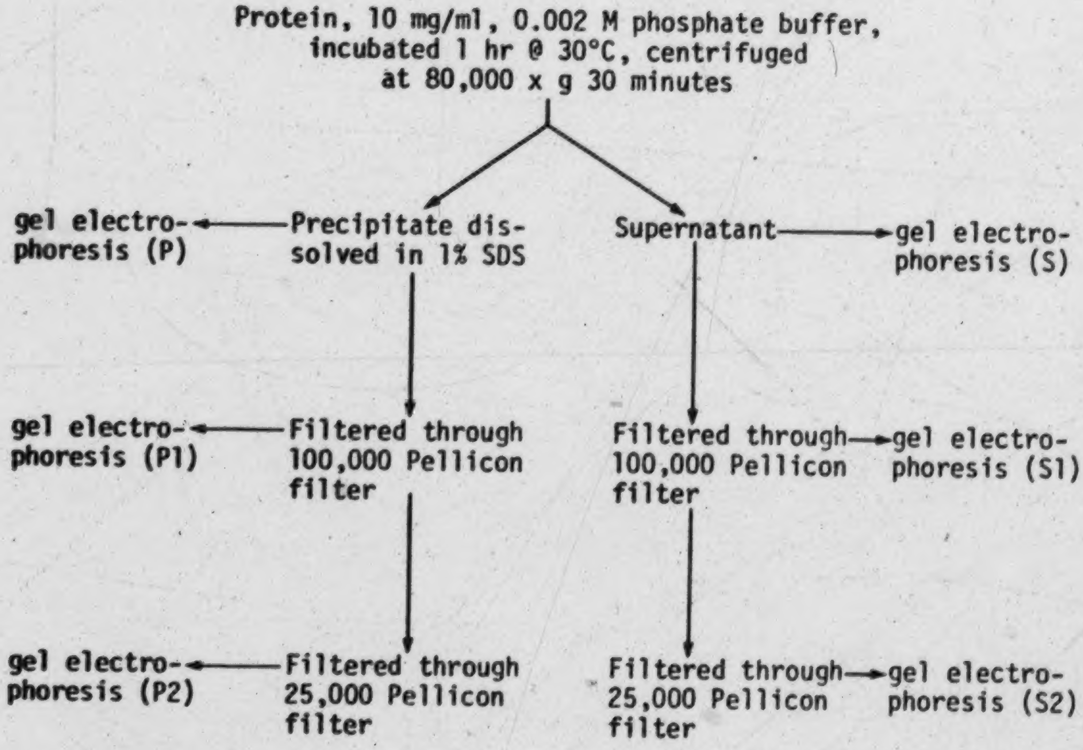
Protein, 10 mg/ml, 0.002 M phosphate buffer, incubated 1 hr @ 30°C, centrifuged at 80,000 x g 30 minutes
gel electrophoresis (P)
Precipitate dissolved in 1% SDS
Supernatant
gel electrophoresis (S)
gel electrophoresis (P1)
Filtered through 100,000 Pellicon filter
Filtered through 100,000 Pellicon filter
gel electrophoresis (S1)
gel electrophoresis (P2)
Filtered through 25,000 Pellicon filter
Filtered through 25,000 Pellicon filter
gel electrophoresis (S2)

Fig. 10. Diagram of the Origin of Samples in Figures 11-23

In text and Figures P and S = unfiltered fraction; P1 and S1=filtrate from 100,000 filter; P2 and S2 = filtrate from 25,000 filter.

definite evidence of proteolysis, the upper band at 34,000 daltons being unchanged α_s-casein and the lower band at 25,000 daltons being a product of proteolysis. In Figure 11eS1 the only band visible at 34,000 daltons was of low intensity. Lysozyme was the only enzyme to give a precipitate with α_s-casein, the precipitate gels (Fig. 12aP, 12bP1, 12cP2) gave a band at 34,000 daltons with perhaps slight reduction of intensity after filtration through the 25,000 filter (Fig. 12cP2). The faint lower band corresponds to the enzyme. The gel of the supernatant after lysozyme treatment, Figure 12dS, gave a band at 34,000 daltons. The gels from β-glucosidase treatment of α_s-casein are shown in Figure 13. The 34,000 dalton band was absent but a strong band appeared at 21,000 daltons, the intensity diminishing after filtration. A diffuse band was apparent at about 11,000 daltons in Figures 13aS and 13bS1.

2. β-casein

The untreated β-casein gels are shown in Figure 14. The precipitate gives a band at 28,000 daltons with unaltered intensity due to filtration (Fig. 14aP, 14bP1, 14cP2). It was not clear whether the apparent band above the main band was in fact separated; it would have a molecular weight of 32,000 daltons. This was the same molecular weight as a low intensity band found in the supernatant gel Figure 14dS. Another very low intensity band was visible at 28,000 daltons in the supernatant gel. Rennin treatment of β-casein gels are shown in Figure 15. The main band of the precipitated protein had a molecular weight of 28,000 daltons and the minor band beneath had a molecular weight of 23,000 daltons. The precipitate appeared to be only slightly affected by filtration. In Figure 15dS only one band at 28,000 daltons was visible in the gel, (poor reproduction responsible for anomolies). The gels of β-casein treated with lysozyme are shown in Figure 16, the intensity of the main band at 28,000 daltons in Figures 16aP, 16bP1 and 16cP2 was not affected by filtration; the lower band was due to lysozyme. In Figure 16dS a faint band at 28,000 daltons was visible in the gel but not in the photographic reproduction. The β-glucosidase treated β-casein gels, Figures 17aP, 17bP1 and 17cP2, showed a major band at 28,000 daltons from the precipitated proteins with a major band at 22,000 daltons and no apparent loss of intensity due to filtration, once again a very faint band from the supernatant protein was visible at 28,000 daltons (Fig. 17dS).

3. κ-casein

The untreated κ-casein gels are shown in Figure 18. The main band visible in the precipitate gels, Figures 18aP, 18bP1 and 18cP2, had a molecular weight of 26,000 daltons. The supernatant gel (Fig. 18dS) had a main band at 26,000 daltons with minor bands at 65,000; 15,000 and 12,000 daltons. Rennin treated κ-casein gels (Fig. 18) showed a main band for the precipitate at 12,000 daltons and a less intense band remaining at 26,000 daltons (Fig. 18eP); a low intensity band at 12,000 daltons was visible in Figure 18fP1. Lysozyme treated κ-casein gels are shown in Figure 19, the small amount of precipitate gave a band at 26,000 daltons with the majority of the protein remaining in the supernatant (Fig. 19dS) giving a band of the same molecular weight. The 12,000 dalton band was attributed to the enzyme. β-glucosidase treatment of κ-casein gave a non-filterable band in the precipitate fraction (Fig. 19eP) at 26,000 daltons and another more intense band at 12,000 daltons. These bands were also visible in the supernatant sample (Fig. 19fS) although the 12,000 dalton band was the less prominent.

4. Calcium Caseinate

A total of eight clearly defined bands were visible in the untreated calcium caseinate precipitate gels (Fig. 20aP, 20bP1, 20cP2), the molecular weights were as shown in the photograph. These bands did not appear to diminish in intensity after filtration. In the supernatant gel only low intensity 35,000 and 27,000 dalton bands were visible (Fig. 20dS). The rennin treated calcium caseinate gels are shown in Figure 21. A decrease in band intensity due to filtration of the precipitate fraction can be seen in Figures 21aP, 21bP1 and 21cP2. In Figure 21dS the gel gave a very faint band at 27,000 daltons not visible in the reproduction. Figure 22 shows the gels of calcium caseinate after lysozyme treatment, no loss of intensity in the bands was apparent after filtration of the precipitate fraction (Fig. 22aP, 22bP1, 22cP2). In the supernatant gel only a very low intensity band was visible at 27,000 daltons (Fig. 22dS). β-glucosidase treatment caused a certain loss of definition of the bands in calcium caseinate precipitate fraction, filtration gave marked lowering of the intensity of these bands also (Fig. 23aP, 23bP1, 23cP2). In the supernatant protein gel the only band was of low intensity at 27,000 daltons.

Figure 11. Control and rennin treated α_s-casein, filtered samples. aS, control supernatant; bS1, filtrate of control supernatant from 100,000 filter; cS2, filtrate of control supernatant from 25,000 filter; dS, supernatant of rennin treated α_s-casein; eS1, filtrate of rennin treated α_s-casein from 100,000 filter. Anode is at the bottom.

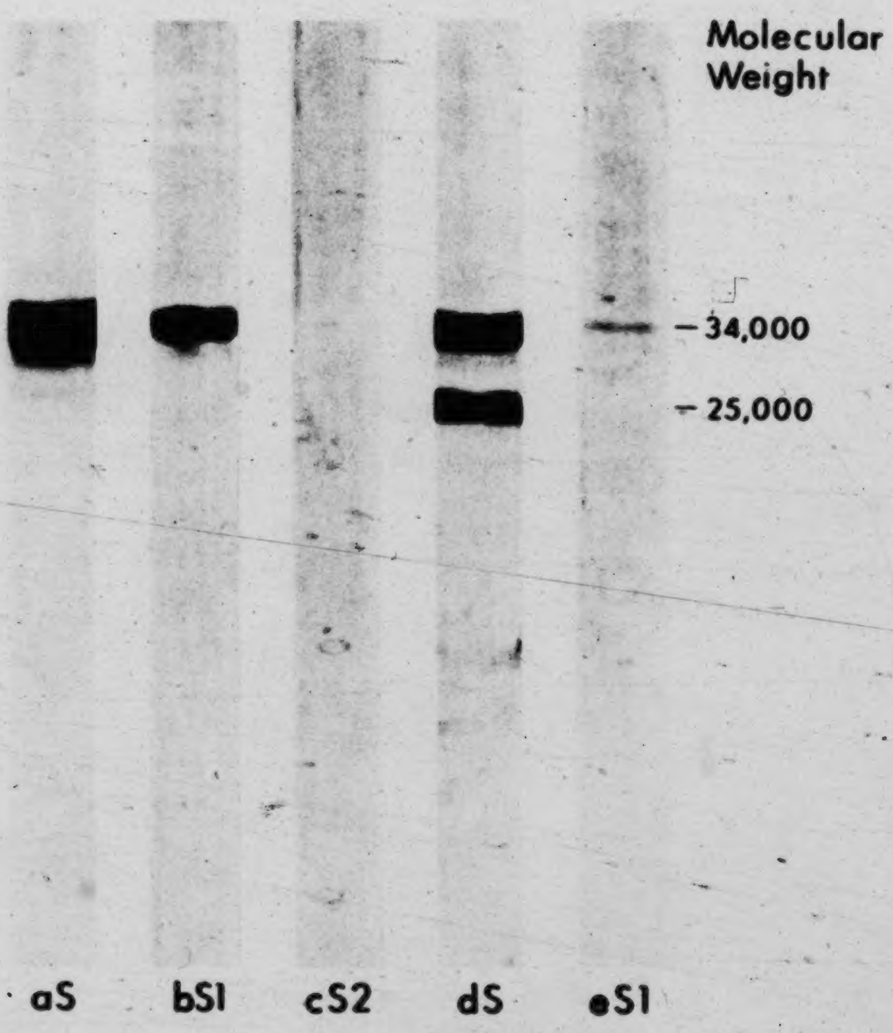

Figure 11

Figure 12. Lysozyme treated α_s-casein, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant. Anode is at the bottom.

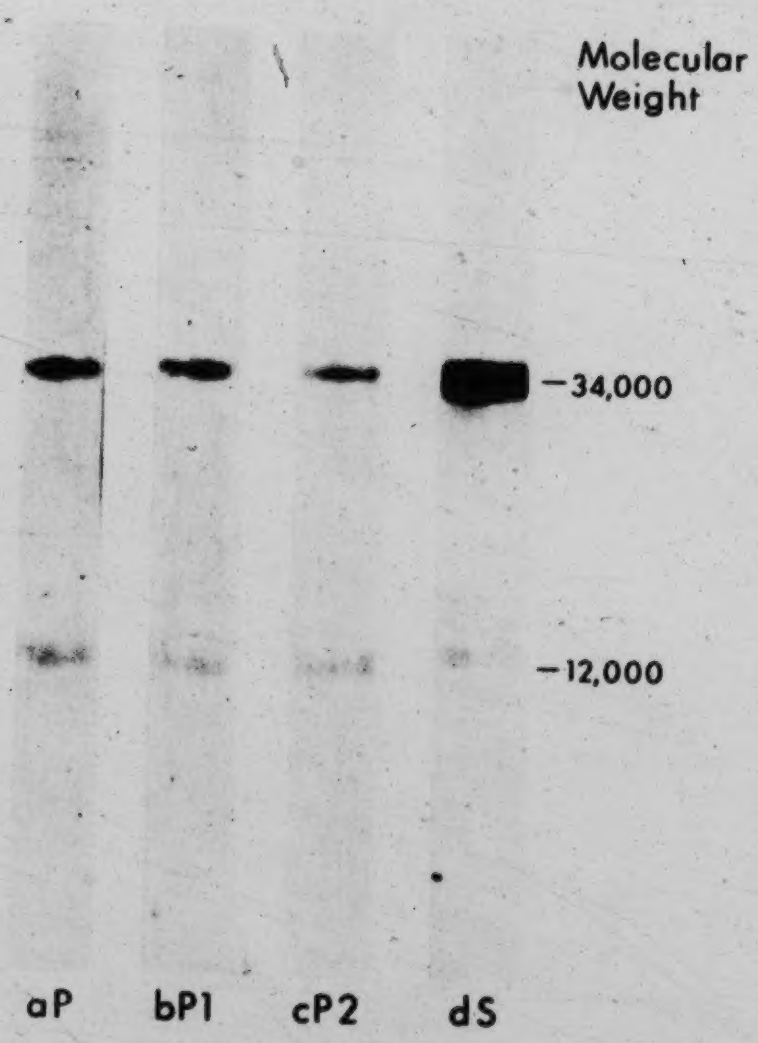

Figure 12

Figure 13. β-glucosidase treated α_s-casein, filtered samples. aS, supernatant; bS1, filtrate of supernatant from 100,000 filter; cS2, filtrate of supernatant from 25,000 filter. Anode is at the bottom.

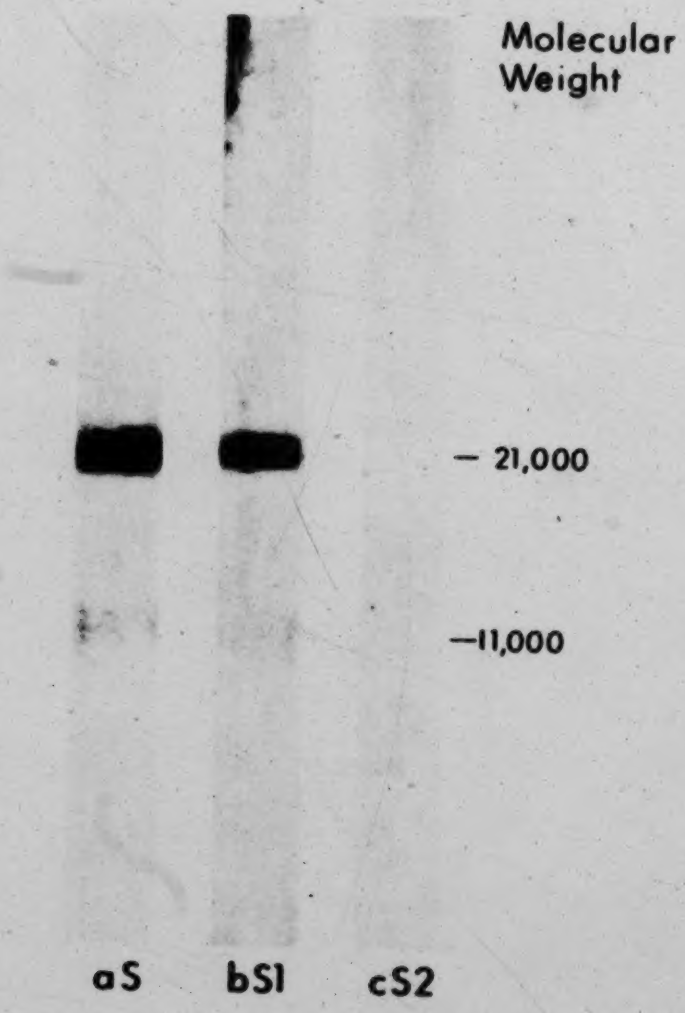

Figure 13

Figure 14. Control β-casein, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant. Anode is at the bottom.

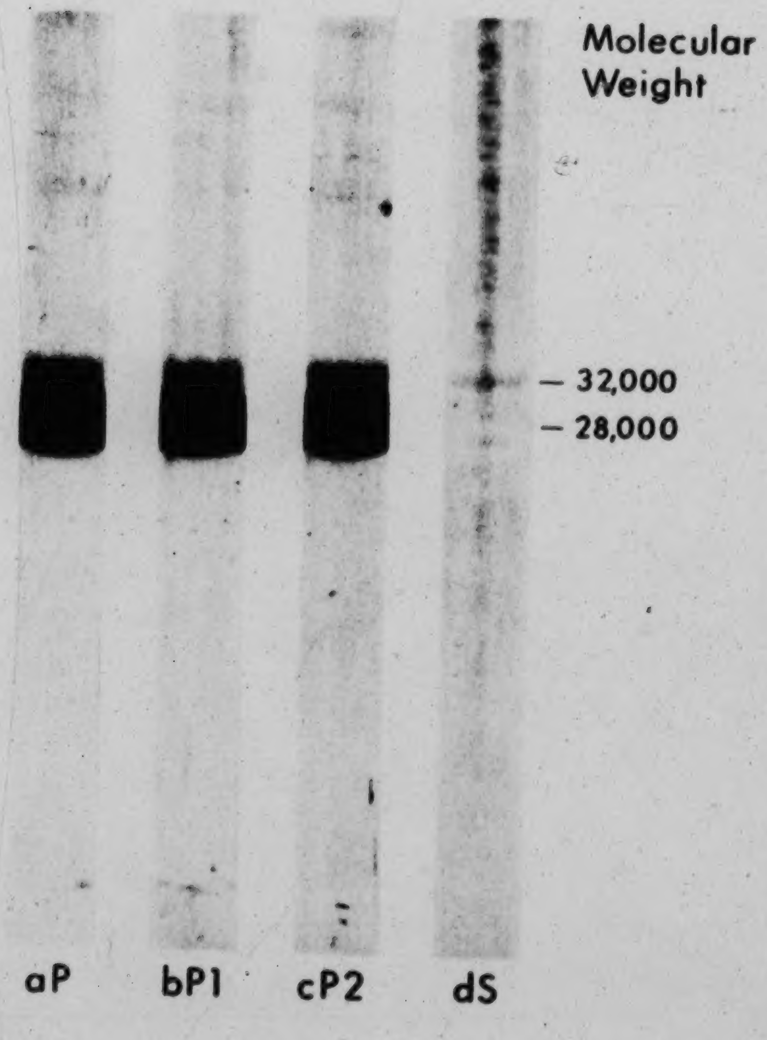

Figure 14

Figure 15. Rennin treated β-casein, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant. Anode is at the bottom.

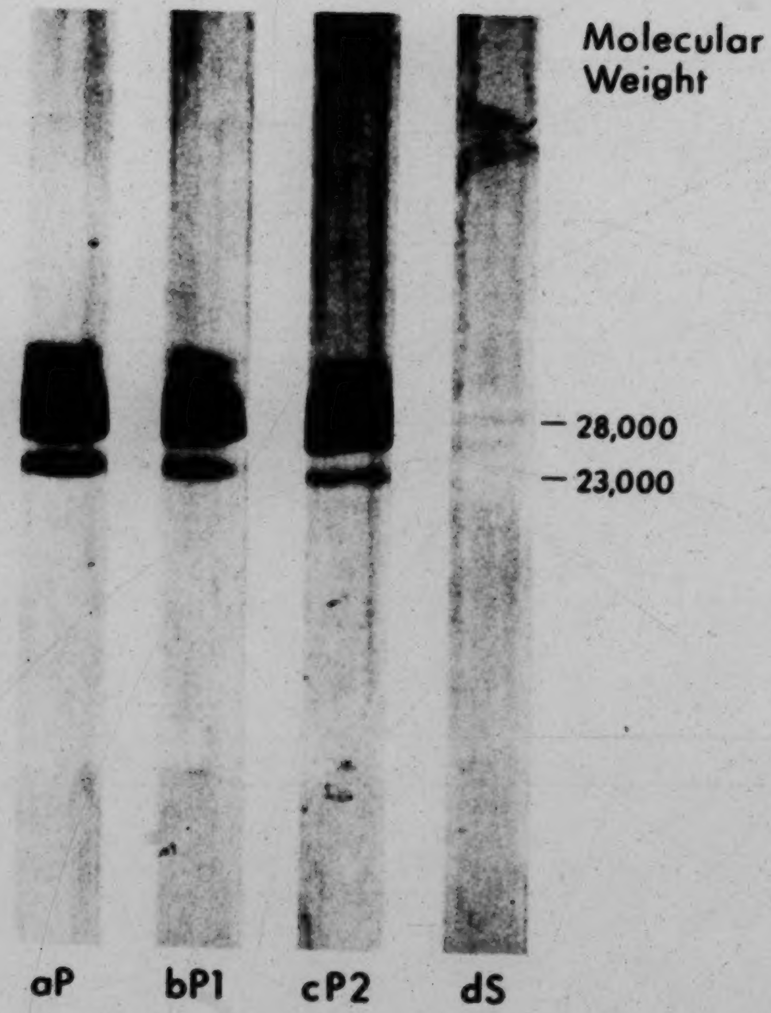

Figure 15

Figure 16. Lysozyme treated β-casein, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant. Anode is at the bottom.

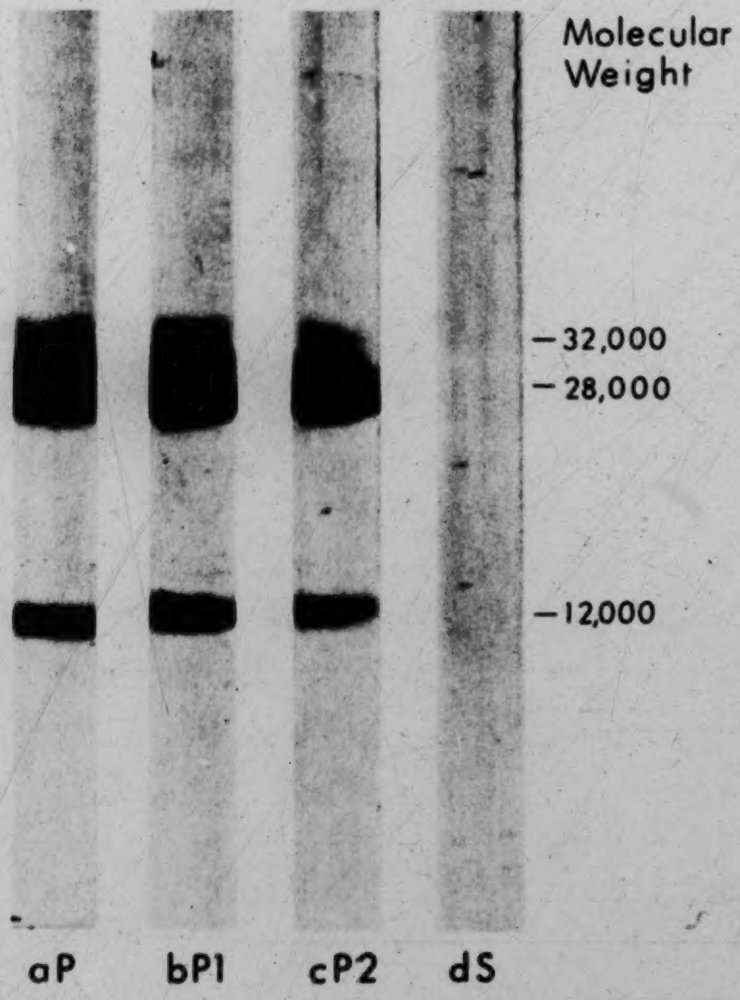

Figure 16

Figure 17. β-glucosidase treated β-casein, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant. Anode is at the bottom.

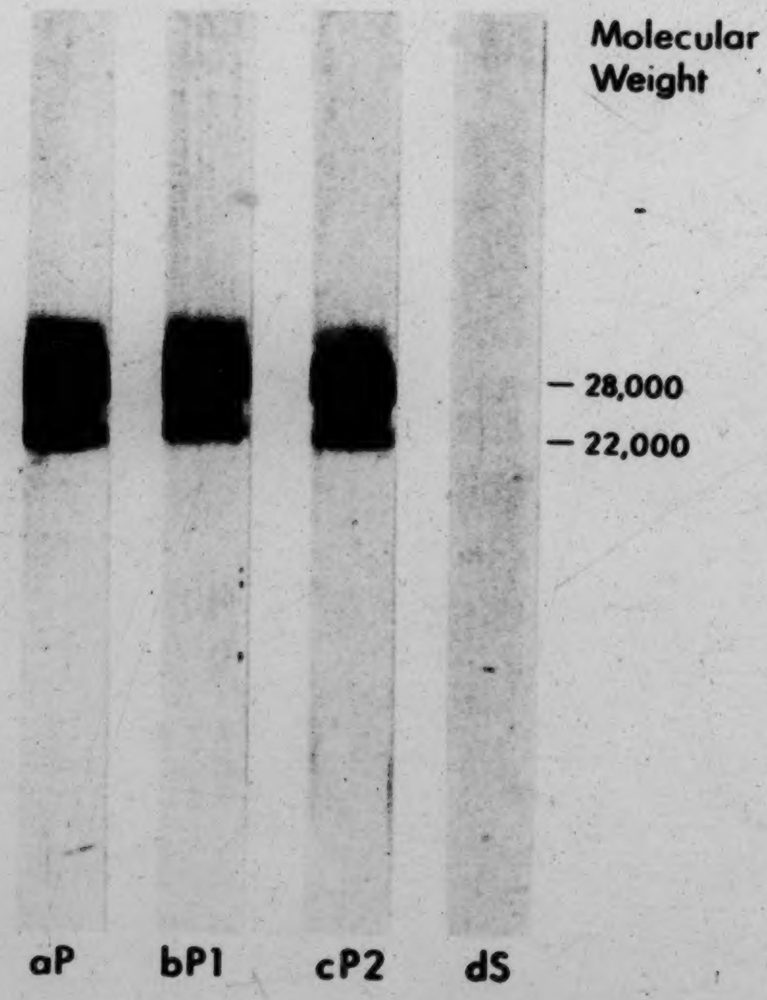

Figure 17

Figure 18. Control and rennin treated κ-casein, filtered samples. aP, control precipitate; bP1, filtrate of dissolved control precipitate from 100,000 filter; cP2, filtrate of dissolved control precipitate from 25,000 filter; dS, control supernatant; eP, precipitate from rennin treatment; fP1, filtrate of dissolved precipitate from rennin treatment from 100,000 filter. Anode is at the bottom.

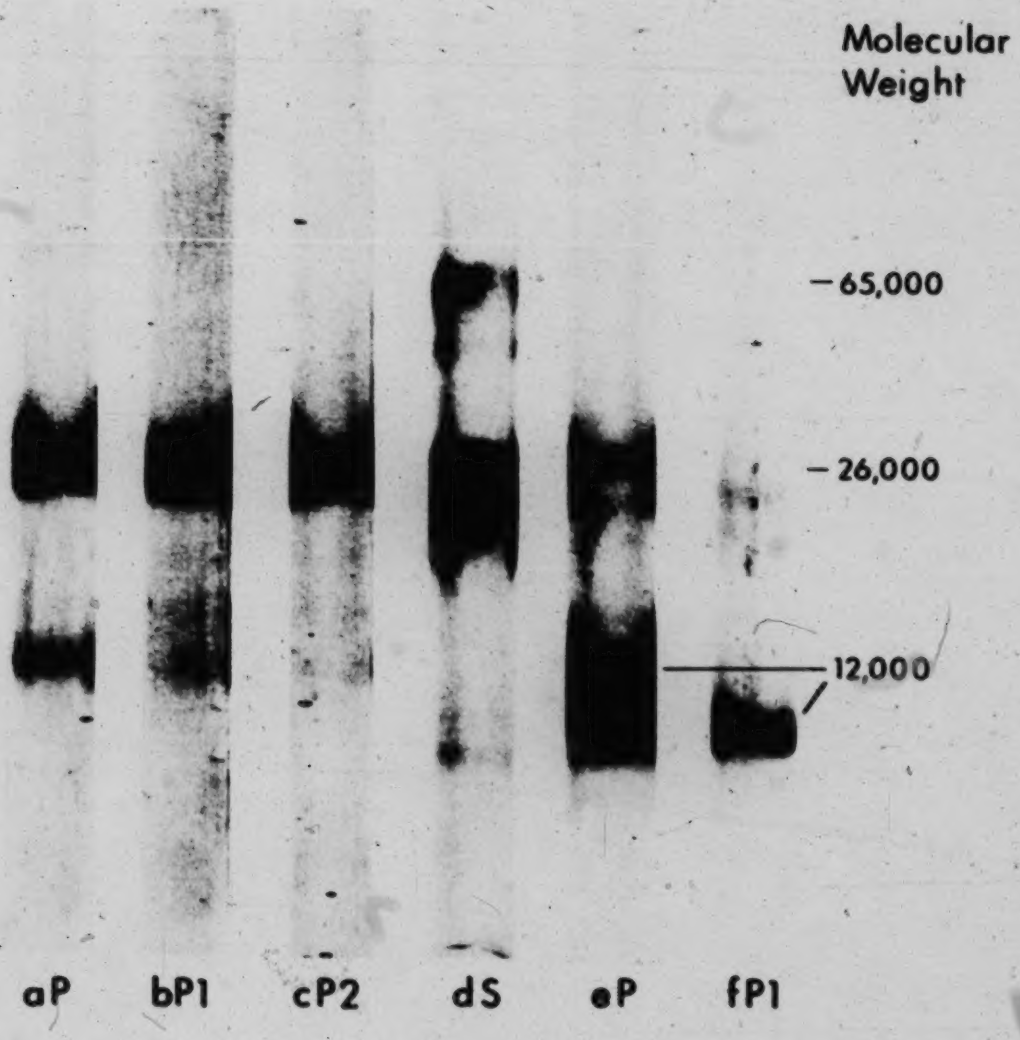

Figure 18

Figure 19. Lysozyme and β-glucosidase treated κ-casein, filtered samples. aP, precipitate from lysozyme treatment; bP1, filtrate of dissolved precipitate from lysozyme treatment from 100,000 filter; cP2, filtrate of dissolved precipitate from lysozyme treatment from 25,000 filter; dS, supernatant from lysozyme treatment; eP, precipitate from β-glucosidase treatment; fS, supernatant from β-glucosidase treatment. Anode is at the bottom.

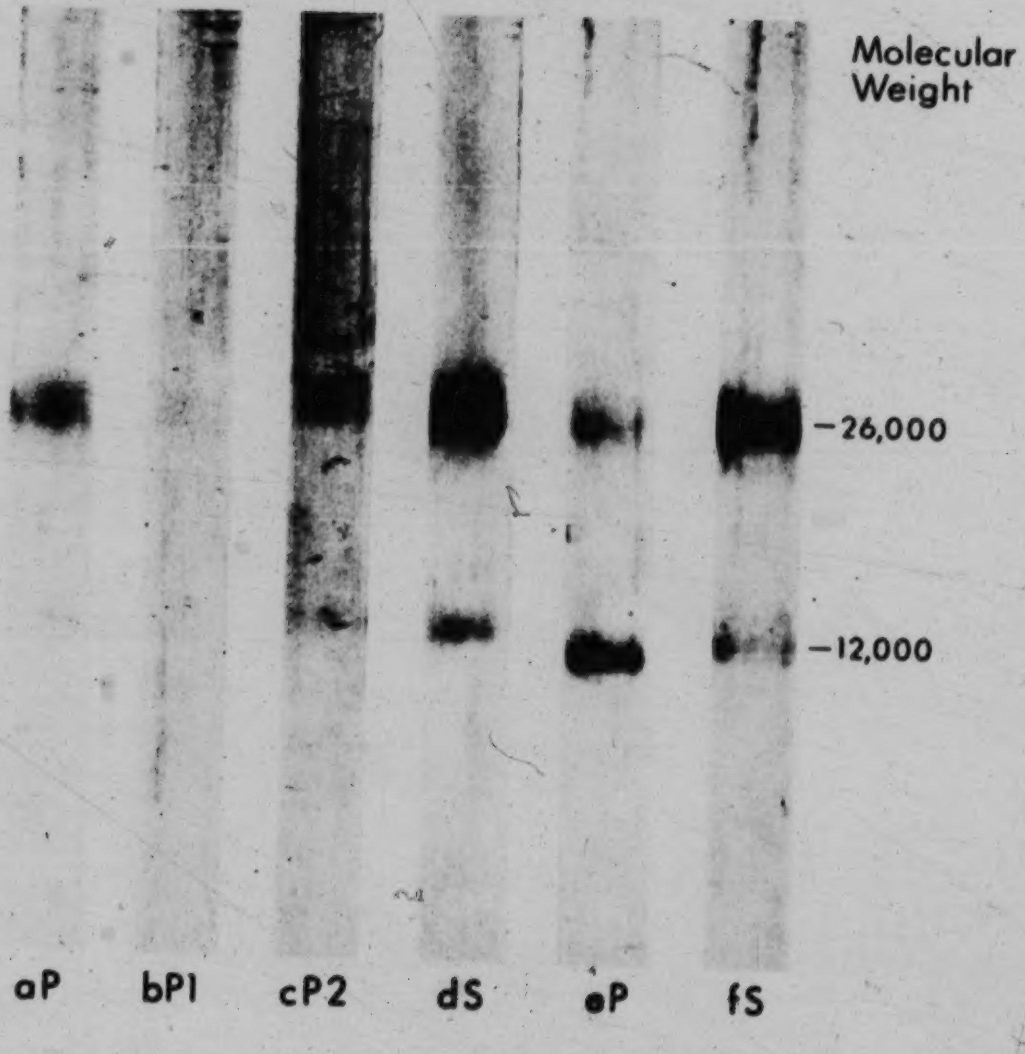

Figure 19

Figure 20. Control calcium caseinate, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant. Anode is at the bottom.

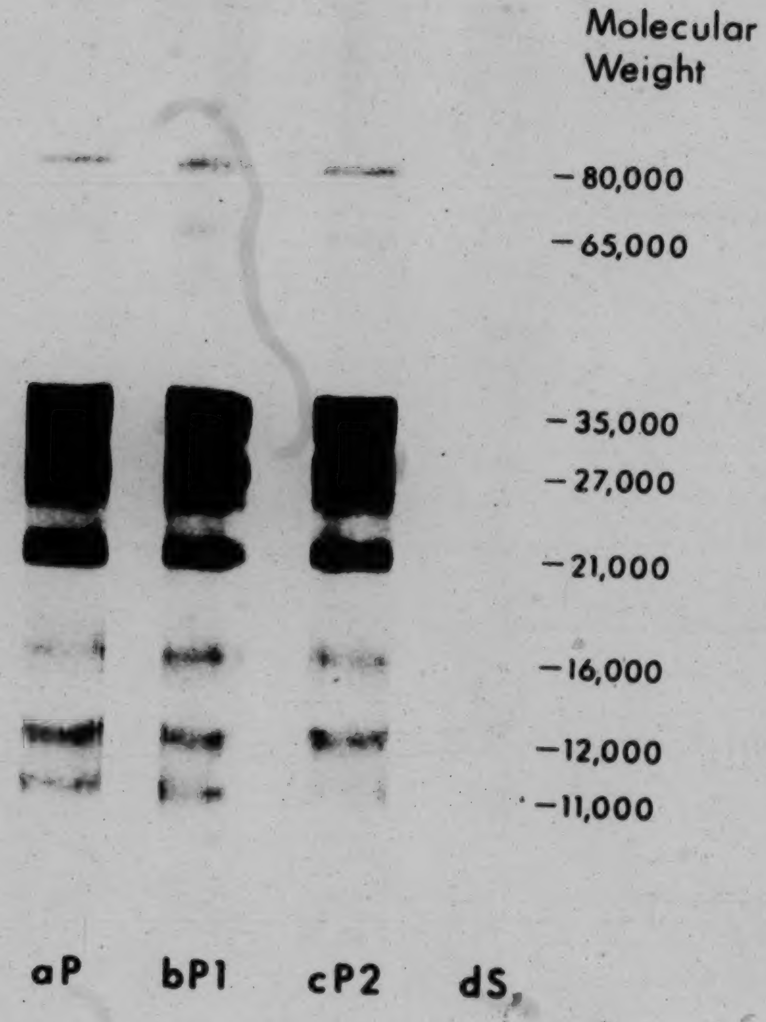
Molecular Weight
– 80,000
– 65,000
– 35,000
– 27,000
– 21,000
– 16,000
– 12,000
– 11,000
aP
bP1
cP2
dS

Figure 20

Figure 21. Rennin treated calcium caseinate, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant. Anode is at the bottom.

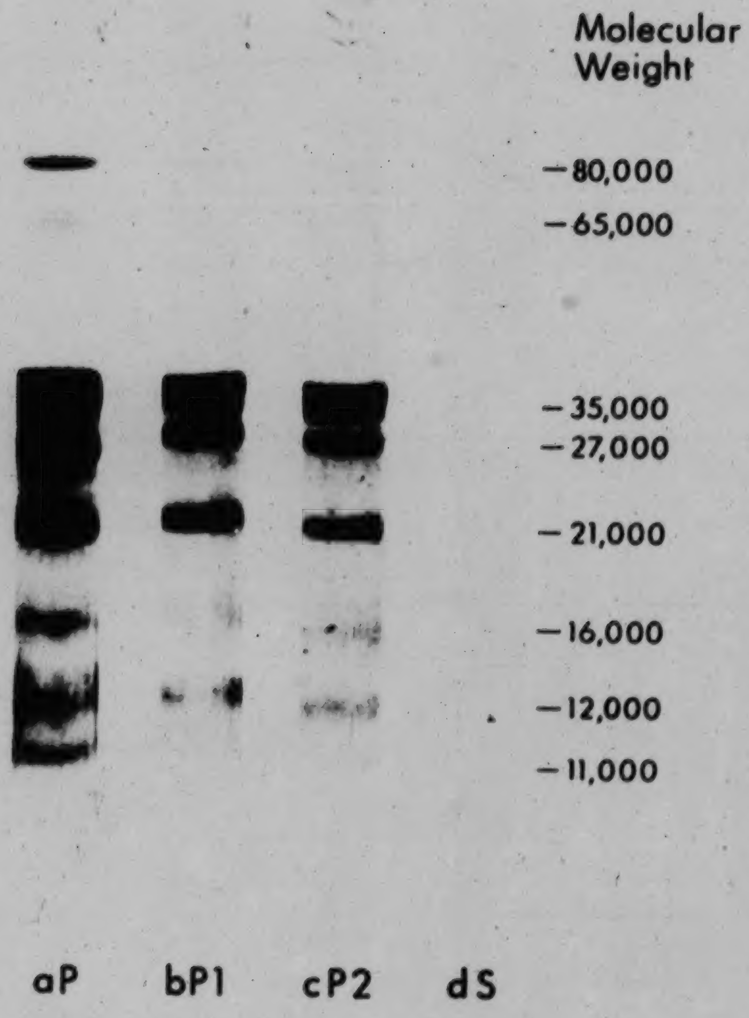
Molecular Weight
— 80,000
— 65,000
— 35,000
— 27,000
— 21,000
— 16,000
— 12,000
— 11,000
aP
bP1
cP2
dS

Figure 21

Figure 22. Lysozyme treated calcium caseinate, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant.

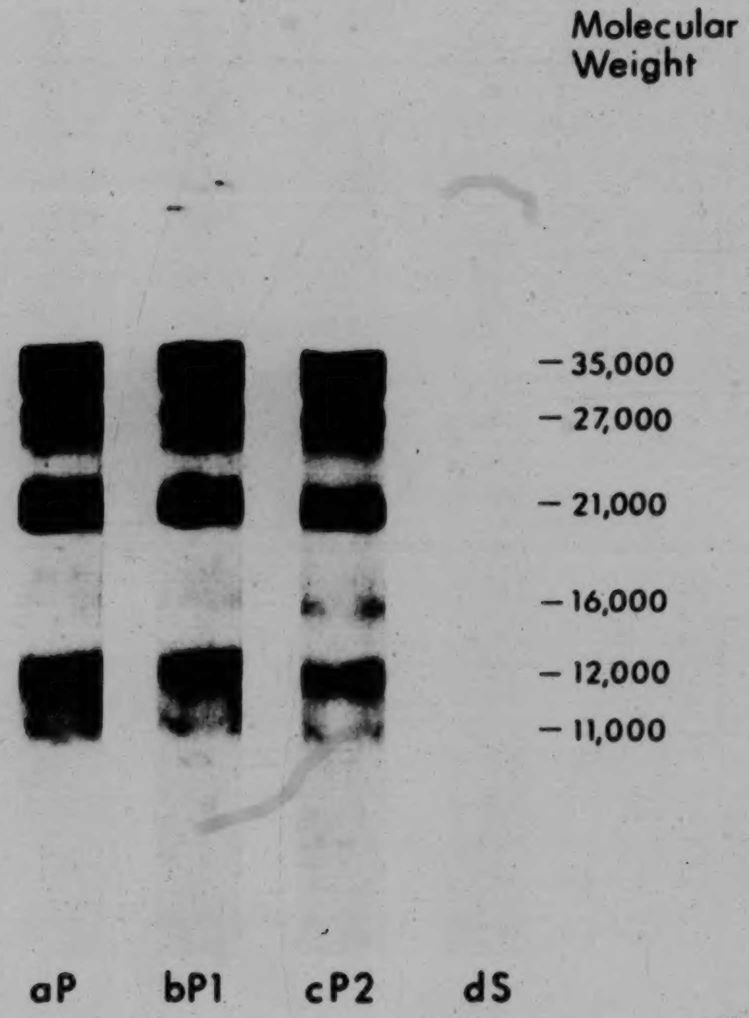
Molecular Weight
— 35,000
— 27,000
— 21,000
— 16,000
— 12,000
— 11,000
aP
bP1
cP2
dS

Figure 22

Figure 23. β-glucosidase treated calcium caseinate, filtered samples. aP, precipitate; bP1, filtrate of dissolved precipitate from 100,000 filter; cP2, filtrate of dissolved precipitate from 25,000 filter; dS, supernatant.

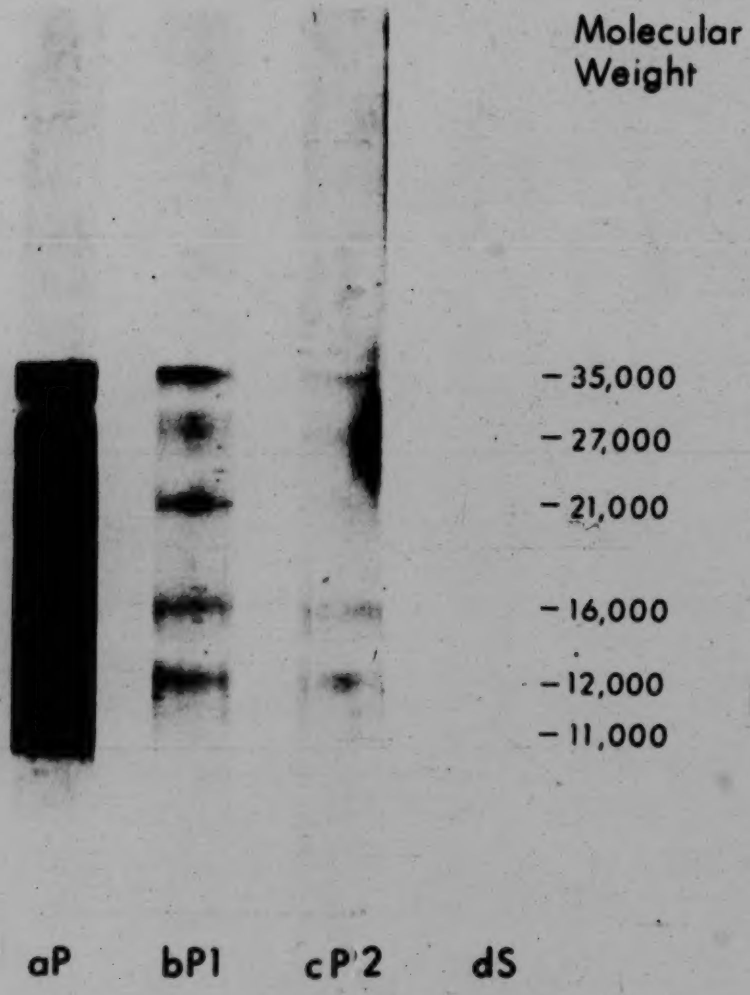
Molecular Weight
– 35,000
– 27,000
– 21,000
– 16,000
– 12,000
– 11,000
aP
bP1
cP2
dS

Figure 23

F. Nitrogen Soluble in Trichloracetic Acid

To amplify the results obtained from the gel electrophoresis work the amounts of nitrogen soluble in 2% and 12% TCA after treating the casein fractions with enzymes were investigated. The increase in the amount of soluble nitrogen in 2% TCA is due to protein fragments or strongly hydrophilic protein fractions. In 12% TCA only proteins or protein fragments of a highly hydrophilic nature will be soluble, such as the carbohydrate bearing fragment of κ-casein after rennin treatment (Nitschmann and Bohren, 1955; Nitschmann and Beeby, 1960). Since the relatively new method of nitrogen determination using the ammonia electrode was used, a comparison of the results with the titration method was carried out using a standard solution of bovine serum albumin. The results are summarized in Table 4. The two methods of analysis were found to be equal in accuracy; in practice the electrode method was more accurate at low concentrations of soluble nitrogen in TCA. The volume of the TCA samples was 2 ml and the accuracy of the analysis of soluble nitrogen was taken to the second decimal place in mg/ml. The results of the analysis are summarized in Tables 5 and 6. In all cases 20 mg of protein was used (2 ml of protein at 10 mg/ml) and after addition of TCA a total of 3 ml was available for analysis. After centrifugation and filtration 2 ml was recovered for nitrogen analysis. The results were calculated on the basis of the total amount of soluble nitrogen per 20 mg of protein and tabulated in this way. Direct comparison can be made between the effect of the enzymes on a particular protein fraction; a histogram (Fig. 24) illustrates the effects of rennin and β-glucosidase on the proteins.

TABLE 4

COMPARISON OF NITROGEN FOUND BY TITRATION AND ELECTRODE METHODS USING STANDARD SOLUTION OF BOVINE SERUM ALBUMIN

Nitrogen added (mg)*	Nitrogen found by titration (mg)	Nitrogen found by electrode (mg)
5.00	4.81	4.75
2.50	2.54	2.55
1.00	1.02	1.00
0.50	0.50	0.53
0.25	0.23	0.26
0.02	0.02	0.03

*Nitrogen calculated at 6.25% of total weight of bovine serum albumin

TABLE 5

AMOUNT OF NITROGEN (mg) SOLUBLE IN 2% TRICHLORACETIC ACID AFTER ENZYME TREATMENT OF PROTEINS (20 mg)

		Incubation Time (mins)				
	Treatment	0	15	30	60	120
α_s-casein	None	0.04	-	0.03	-	0.03
	Rennin	0.03	0.04	0.05	0.04	0.06
	Lysozyme	0.04	0.03	0.04	0.03	0.03
	*β-glucosidase	0.04	0.07	0.10	0.15	0.24
β-casein	None	0.04	-	-	-	0.03
	Rennin	0.04	0.03	0.04	0.07	0.06
	Lysozyme	0.03	0.04	0.04	0.03	0.04
	β-glucosidase	0.04	0.04	0.06	0.05	0.07
κ-casein	None	0.12	0.14	0.14	0.14	0.15
	*Rennin	0.15	0.40	0.47	0.71	0.82
	*Lysozyme	0.14	0.12	0.14	0.15	0.17
	*β-glucosidase	0.14	0.12	0.16	0.17	0.22
Calcium-Caseinate	None	0.04	0.04	0.04	0.04	0.04
	Rennin	0.04	0.05	0.07	0.08	0.10
	Lysozyme	0.03	0.03	0.04	0.04	0.04
	β-glucosidase	0.04	0.06	0.06	0.06	0.09

*Average of two determinations, all other results from one determination

Proteins incubated with enzymes at pH 6.3 for 1 hr at 30°C

TABLE 6

AMOUNT OF NITROGEN (mg) SOLUBLE IN 12% TRICHLORACETIC ACID AFTER ENZYME TREATMENT OF PROTEINS (20 mg)

	Treatment	Incubation Time (mins)				
		0	15	30	60	120
α_s-casein	None	0.03	0.03	0.03	0.04	0.03
	Rennin	0.03	0.03	0.03	0.03	0.03
	Lysozyme	0.04	0.03	0.03	0.03	0.03
	*β-glucosidase	0.04	0.06	0.05	0.06	0.07
β-casein	None	0.04	-	-	-	0.04
	Rennin	0.03	0.04	0.04	0.04	0.04
	Lysozyme	0.04	0.03	0.04	0.03	0.05
	β-glucosidase	0.05	0.04	0.06	0.04	0.05
κ-casein	None	0.10	-	-	-	0.10
	*Rennin	0.10	0.18	0.24	0.27	0.30
	*Lysozyme	0.08	0.08	0.09	0.10	0.09
	*β-glucosidase	0.10	0.10	0.11	0.10	0.11
Calcium-caseinate	None	0.04	0.03	0.03	0.03	0.04
	Rennin	0.03	0.04	0.04	0.05	0.06
	Lysozyme	0.04	0.03	0.03	0.03	0.03
	β-glucosidase	0.04	0.04	0.05	0.04	0.04

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*Average of two determinations, all other results from one determination

Proteins incubated with enzymes at pH 6.3 for 1 hr at 30°C

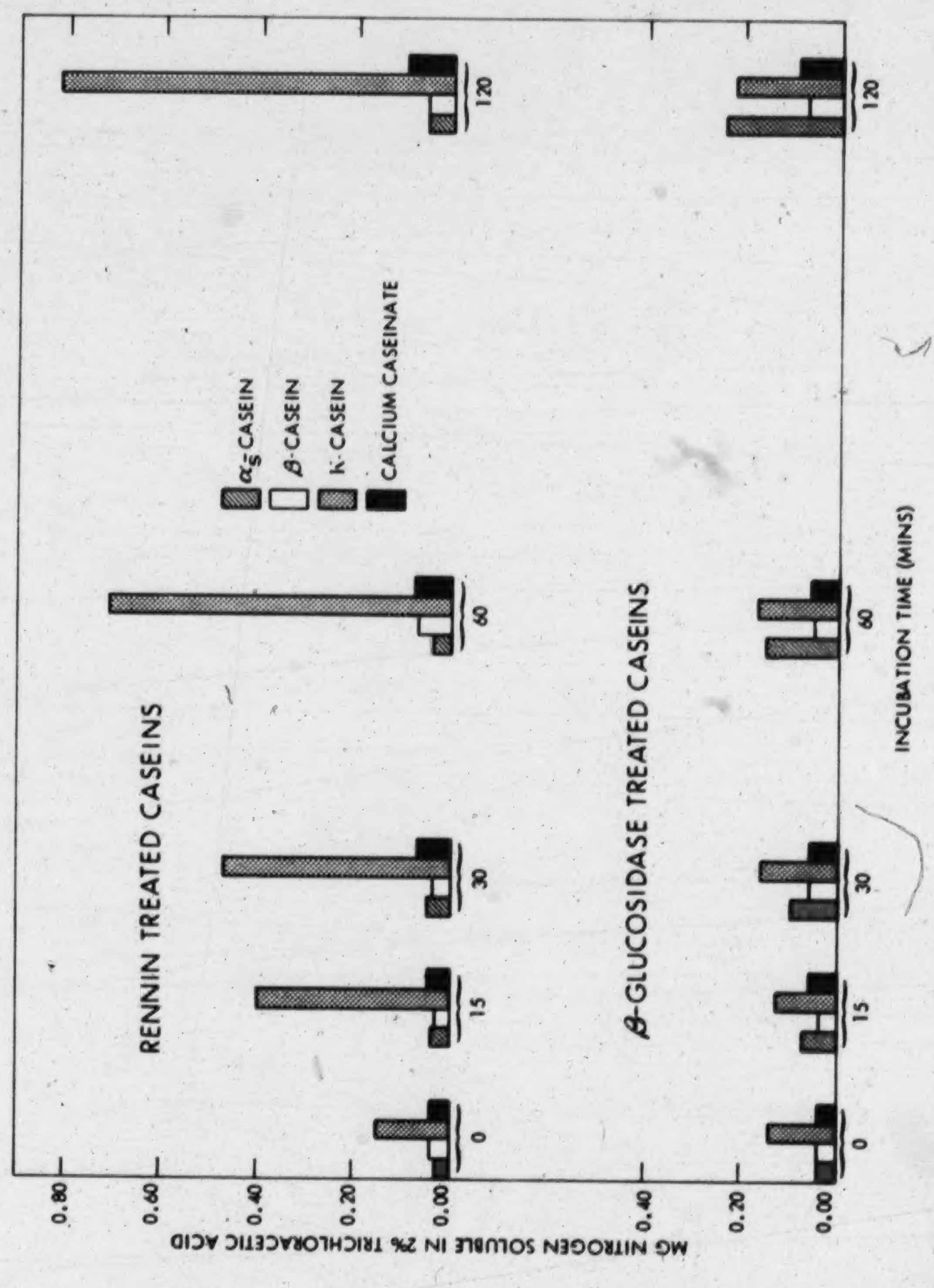

Figure 24. TCA soluble nitrogen from rennin and β-glucosidase treatment of caseins

Blank determinations were consistent throughout; with no enzyme added the proteins did not show an increase in soluble nitrogen with respect to time, though the amount derived from κ-casein was higher than found in the other casein fractions. In 2% TCA rennin showed slight increases in the amount of soluble nitrogen from α_s-casein and calcium caseinate, κ-casein gave a considerable amount as expected. The amount soluble in 12% TCA after rennin treatment was low from α_s-casein with a slight increase in calcium caseinate; the hydrophilic macroglycopeptide was responsible for the appreciable amount in the case of κ-casein after 2 hours incubation. No definite trend in the increase of soluble nitrogen could be found after lysozyme treatment of the proteins in either the 2% or 12% TCA. In 2% TCA the β-glucosidase treated caseins all showed some increase in the amount of soluble nitrogen, α_s- and κ-casein to a greater degree than the other two fractions. In 12% TCA only α_s-casein showed a slight increase in soluble nitrogen after β-glucosidase treatment.

G. Gas Chromatography

1. Standards

To attempt identification of carbohydrates split off the caseins by glycolytic enzymes, the derivatives of a number of standards were prepared. In Table 7 the carbohydrates, the relative retention volumes of elution with respect to the pyranose form of α-D-galactose derivative and the probable structural form of some of the isomers are shown, together with the relative molar detector response with respect to D-galactose. Solutions of sucrose, maltose and α-lactose were dried and derivatized leaving out the methanolic hydrolysis and re-N-acetylation steps. The position of these three disaccharides on the chromatogram were defined in

TABLE 7

RELATIVE RETENTION TIMES OF STANDARDS WITH RESPECT TO THE α-PYRANOSE ISOMER OF D-GALACTOSE; RELATIVE MOLAR DETECTOR RESPONSE WITH RESPECT TO RESPONSE OF D-GALACTOSE

Methyl Glycoside	Isomer(1)	Relative Retention Time	Relative Molar Response
L-Rhamnose (6-Deoxy-L-mannose)		0.66	1.13
L-Fucose (6-Deoxy-L-galactose)	f	0.64	
	α-p	0.66	1.02
	β-p	0.88	
D-Mannose		0.94	0.92
D-Galactose	f	0.97	
	α-p	1.00	1.00
	β-p	1.03	
D-Galactosamine (2-Amino-2-deoxy-D-galactose)		1.05	0.80
D-Glucose	α-p	1.05	0.98
	β-p	1.08	
D-Glucosamine (2-Amino-2-deoxy-D-glucose)		1.10	0.77
		1.17	
N-Acetyl-D-galactosamine (2-Acetamido-2-deoxy-D-galactose)		1.31	0.63
N-Acetyl-D-glucosamine (2-Acetamido-2-deoxy- D-galactose)	β-p	1.33	0.48
	α-p	1.39	
Neuraminic acid (Sialic acid)		1.68	0.15
Sucrose		2.26	
Lactose	α	2.21	
	β	2.32	
Maltose		2.69	

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(1) Isomer designations from Clamp et al. (1967) and Sweeley and Vance (1967). f = furanose; α-p = α-pyranose; β-p = β-pyranose

order to check that the methanolic hydrolysis procedure had reached completion when analyzing the casein samples. All other samples were refluxed in methanolic HCl but no re-N-acetylated; except for sialic acid all derivatives were formed from the methyl glycosides, i.e., the -OH in the C-1 position was converted to an $-OCH_3$ group (Fig. 25). The structure of sialic acid after methanolysis and silylation is shown in Appendix III (Sweeley and Vance, 1967).

The re-N-acetylation procedure according to Clamp et al. (1967) was attempted on D-galactosamine but results were erratic and not reliable enough for quantitative work, no reason could be found for the problems encountered although high purity reagents were used throughout. Methanolysis of N-acetyl-galactosamine and N-acetyl-neuraminic acid for 2 and 6 hours gave the same quantitative results, no D-galactosamine was produced and no shift in the retention volume of N-acetyl neuraminic acid could be detected. Wheelock and Sinkinson (1969) postulated that the N-acetyl groups were not removed during methanolic hydrolysis of milk glycopeptides although no quantitative details were given in the report. In this project, without using re-N-acetylation, a considerable portion of the D-galactosamine was found as the amine and not N-acetylated. The amount of N-acetyl-D-galactosamine after conversion to the equivalent amount of D-galactosamine was combined with the amount of free amine and reported as total D-galactosamine.

The monosaccharides found in κ-casein were identified by retention volume and then by injecting known standards together with the carbohydrate derivatives of κ-casein. The internal standards increased the respective peak areas and heights of the κ-casein derived peaks under

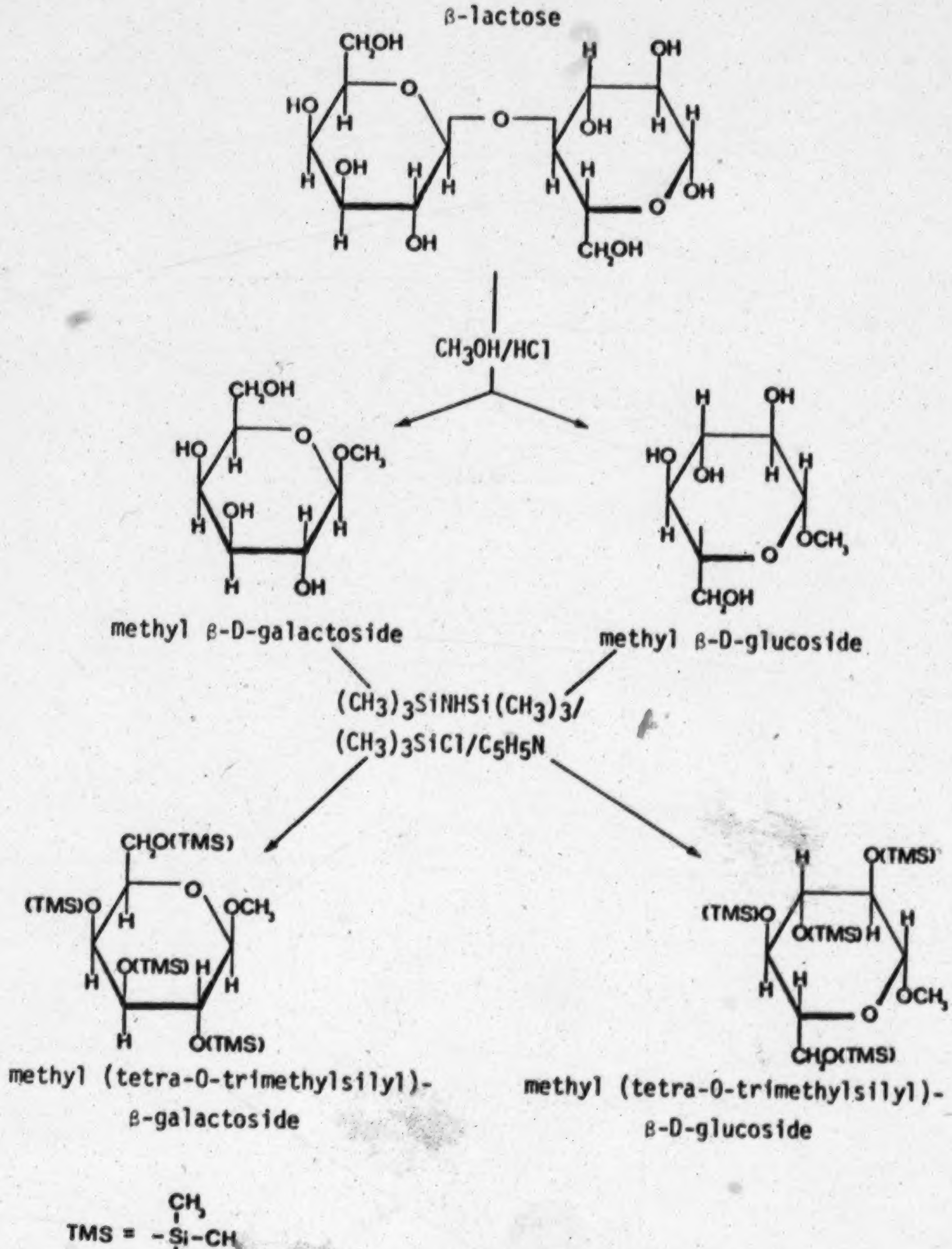

Figure 25. Formation of the trimethylsilyl derivatives of β-lactose after methanolic hydrolysis

scrutiny. The sensitivity range used on the chromatograph was 10^{-11} amps/mV, the usual attenuation was 1/64, attenuations below this caused problems due to tailing from the solvent and excess reagent peak and spurious baseline fluctuations due to artifacts from hydrolysis, derivatization and liquid phase degradation. At this level 1×10^{-6}g of D-galactose gave a major peak of ≈100 mm in height though the minor peaks were hard to quantitate. For the quantitative work higher attenuations were regularly used and larger amounts of derivatives introduced. The volume injected was kept to a minimum to avoid the build up of products of pyrolysis on the detector collector ring and the detector housing, the volume was always 5 µl or less. Larger injection volumes also caused excessive tailing of the injection peak and bleeding of the liquid phase, giving a series of spurious peaks which could be confused with carbohydrate derivatives at low concentrations. The peak areas were calculated by triangulation, a baseline was drawn at the base of the peak continuing the normal baseline, tangents were drawn to the most linear portions of the upslope and downslope and continued to intersect the baseline and to intersect themselves above the peak apex.

2. Carbohydrate Contamination of Enzymes

Rennin and β-glucosidase after methanolic hydrolysis and derivatization were found to be contaminated with carbohydrates; no carbohydrates could be detected from lysozyme. Rennin was contaminated with D-galactose and D-glucose in equal proportions, the total was calculated to be 66% by weight. With the amount of rennin added to the proteins the carbohydrate contamination was significant and the calculated amount had to be subtracted from the amount due to other sources. In the case of β-glucosidase the contaminants were D-mannose and D-galactose; the total amount was 7% by weight, 62% of this due to D-mannose and 38% due to D-galactose. In the amounts used in the protein treatment these carbohydrates were not detectable and no corrections were made in calculations of carbohydrates from β-glucosidase treated proteins. The initial qualitative work was designed to identify any carbohydrates released from the casein fractions due to enzyme action. The caseins were treated with the enzymes, heated in a boiling water bath for ten minutes to inactivate enzymes then placed in a dialysis bag and dialyzed for 48 hours against 4 litres of water. The dialysate was then evaporated down to 25 ml and portions dried, hydrolyzed and derivatized. Inconsistent results were obtained, the reproducibility of results was very poor after much time had been spent trying to improve the method it was abandoned in favor of filtration methods. It was thought that contamination could occur during dialysis and that the evaporation procedure may lead to reaction of products of the enzyme treatment.

3. Gas Chromatography of the Filtered Products of Enzyme Treated Caseins

The experimental scheme is shown in Figure 26. A qualitative study was carried out initially, the highest sensitivity possible was used on the chromatograph, 10^{-11} amps/mV x 1/8 and x 1/4. The baseline problems in these ranges were serious but major peaks could be recognized and were identified. Lower sensitivities were used for quantitative work and standards were prepared of approximately the same concentration as encountered from the carbohydrates derived from the caseins. Detector

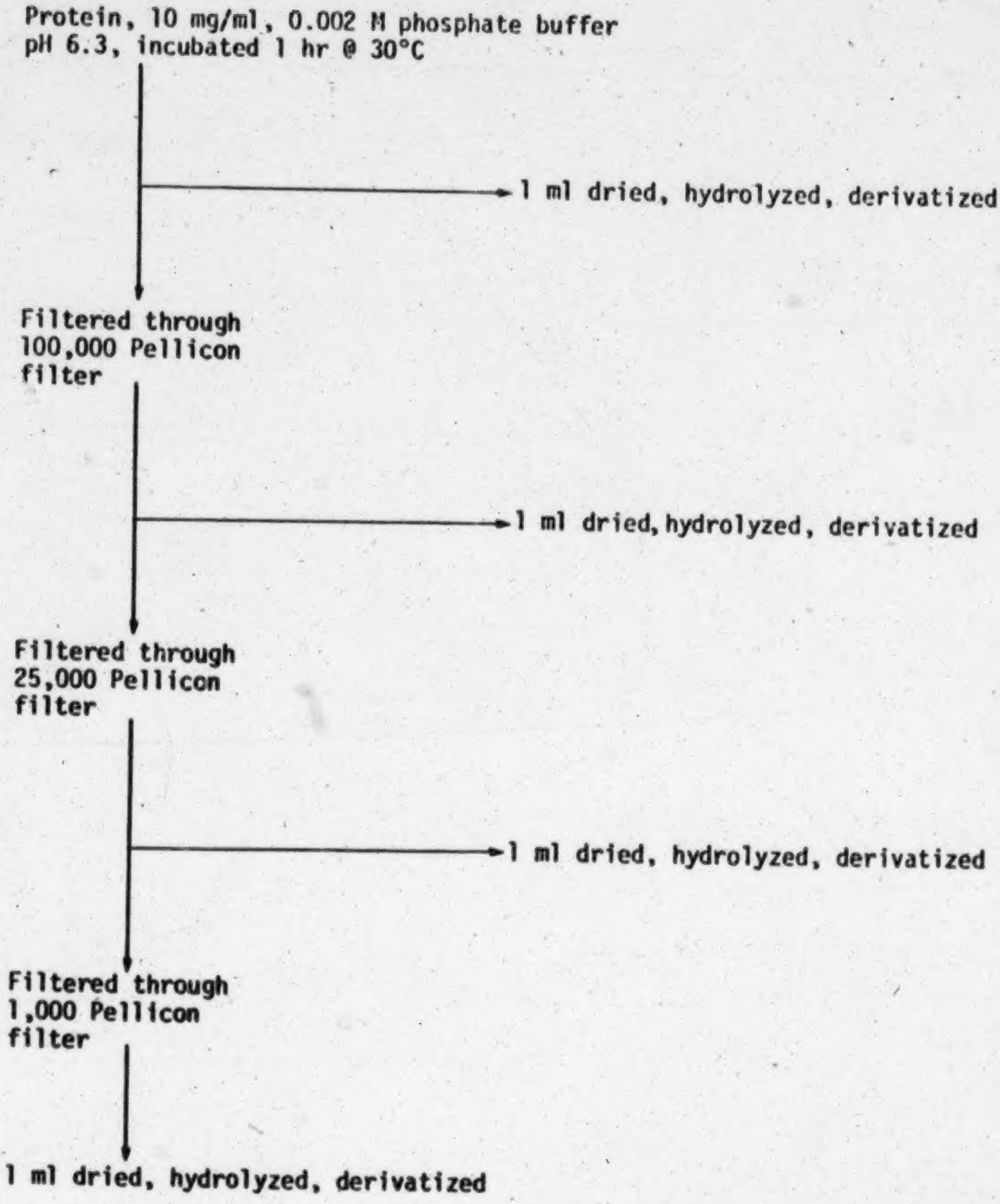
Protein, 10 mg/ml, 0.002 M phosphate buffer
pH 6.3, incubated 1 hr @ 30°C
1 ml dried, hydrolyzed, derivatized
Filtered through 100,000 Pellicon filter
1 ml dried, hydrolyzed, derivatized
Filtered through 25,000 Pellicon filter
1 ml dried, hydrolyzed, derivatized
Filtered through 1,000 Pellicon filter
1 ml dried, hydrolyzed, derivatized

Figure 26. Diagram of the origin of samples for carbohydrate analysis

response of the standards was found to be linear over the narrow ranges required. Problems were encountered in the determination of D-galactosamine, N-acetyl-D-galactosamine and N-acetyl-neuraminic acid, after two or three samples had been removed from the vial for analysis, the peak areas of these derivatives began to diminish significantly. Since total sample volume was 0.4 ml the effects of opening the vial and allowing air and moisture to enter affected the stability of the derivatives. The standards of these carbohydrates were made up in 2 ml of the derivatizing mixture and were stable for at least two days even after removing up to six samples per day. Derivatives of D-galactose, and D-glucose and D-mannose were not affected to the same degree and were stable after the amino sugar derivatives had deteriorated.

4. α_S- and β-caseins

No carbohydrates could be detected after methanolic hydrolysis and derivatization of the α_S- or β-caseins. Enzyme treatment did not release any detectable carbohydrates.

5. κ-casein

According to the work of Huang et al. (1964), κ-casein prepared by the method of Zittle and Custer (1963) contains 29 mg of carbohydrates per g protein. The combined molecular weights of sialic acid, 298 daltons; D-galactose, 180 daltons and D-galactosamine, 179 daltons give a combined total of 657 daltons. Assuming the molecular weight of κ-casein to be 20,000 daltons this total represents 33 mg carbohydrates per gram of κ-casein. Alais and Jolles (1961) found the carbohydrate content of κ-casein, prepared by the method of McKenzie and Wake (1961) to be 50 mg/g. The sialic acid content of κ-casein varies considerably and may be due to the stage of lactation (Mackinlay and Wake, 1971). Using 10 mg of κ-casein the carbohydrate content of the κ-casein used in this study was found to be 23.5 mg/g (Table 8).

Rennin treated κ-casein revealed the same amounts of carbohydrates as were present in the untreated protein. The carbohydrates were able to pass through the 100,000 filter and most through the 25,000 filter. No carbohydrates from κ-casein passed through the 1,000 filter (Table 8).

6. Calcium Caseinate

Calcium caseinate was found to be contaminated with lactose which after hydrolysis and silylation gave derivatives of D-galactose and D-glucose. The total carbohydrate contamination, 21 mg/g, made detection of other derivatives difficult. The amount of D-galactosamine due to κ-casein was masked by the α-D-glucose peak and at high sensitivity recognition of other peaks was impossible due to the poor baseline. Enzyme treatment of the calcium caseinate did not reveal any change in the peak areas of derivatives of the untreated protein, filtration did not cause any reduction in these peaks either which would indicate that the origin of the D--alactose and D-glucose was lactose.

TABLE 8

CARBOHYDRATES FOUND IN κ-CASEIN (mg/g κ-CASEIN)

Total	D-Galactose	D-Galactosamine	Sialic Acid	
50	12	14	24	Alais and Jolles, 1961
28.96	9.2	6.06	13.7	Huang et al., 1964
23.5	10.4	4.3	8.8	This study*

*Average of three determinations (3 replicates per determination)

CARBOHYDRATE ANALYSIS OF FILTERED FRACTIONS FROM RENNIN TREATMENT OF κ-CASEIN (mg/g κ-CASEIN)

Filter Fraction	D-Galactose	D-Galactosamine	Sialic Acid	Total
Unfiltered	10.7	4.8	8.7	24.2
100,000	10.4	4.6	8.2	23.2
25,000	8.2	3.8	5.7	17.7
1,000	0.0	0.0	0.0	0.0

Average of two determinations (3 replicates per determination)

IV. DISCUSSION

As the methods used in this study were adapted and modified from other sources, it was deemed advisable to preface the discussion with a brief discussion of the principal tools. Therefore, the discourse is divided into two sections.

A. Discussion of Methods

SDS gel electrophoresis was used extensively to take advantage of the molecular weight determinations that can be made. The analytical procedure was the same for all proteins studied, not requiring different buffer systems for each casein fraction. SDS gel electrophoresis has been used extensively in the studies of muscle proteins and enzymes (Shapiro *et al*., 1967; Weber and Osborn, 1969; Hay *et al*., 1973), where the bands are generally more discrete than in casein electrophoresis due to the homogeneity of the proteins. The heterogeneous caseins would be expected to form broader bands, as seen in the casein fraction gels, and this causes problems in interpretation as seen in the calcium caseinate gels. Only tentative identification of the major casein fractions can be made since standards of purified caseins are not readily available. It would be interesting to check the purity of α_s-, β- and κ-caseins used in this study with highly purified preparations of other workers.

Preparative SDS gel electrophoresis could be employed to investigate the composition of the casein preparations and degradation products which result from enzyme action. This would lead to the indentification of the enzyme sensitive bonds and the composition of the fragments.

It was not possible to make any mathematical calculations to account for the origins of the degradation products, i.e., the addition of the molecular weights of units of the degradation products does not coincide with the molecular weight of the parent pure proteins. This may be due to the uncertainty of the molecular weights as calculated from the gels; the abnormal migration of lysozyme is an example of this problem (Dunker and Rueckert, 1969). Since very consistent results were obtained, possibly rates of degradation could be calculated using a densitometer to obtain quantitative results. Problems occur, however, if the stainability of the degradation products is different from the parent material. None of these problems are insurmountable and preparative as well as analytical SDS gel electrophoresis should prove to be of great value in milk protein research due to its simplicity and versatility.

The trichloracetic acid soluble nitrogen phase of the work was carried out in order to amplify the results of the gel electrophoresis. The use of the recently available ammonia specific ion electrode was certainly rewarding and without doubt, this method saved a lot of time and gave consistent results.

In studies of the degradation of whole milk and casein fractions by rennin the measurement of TCA soluble nitrogen as a parameter of the progress of the reaction has been established (Nitschmann and Bohren, 1955; Nitschmann and Beeby, 1960). In 2% TCA peptides and hydrophilic protein fractions are soluble, in 12% TCA only highly hydrophilic macropeptides are soluble. The only casein fraction soluble in 12% TCA is the macroglycopeptide, the attached trisaccharide being responsible for the additional hydrophilicity.

The analysis of carbohydrates by gas chromatography using the volatile trimethylsilyl derivatives was chosen in order to give both quantitative and qualitative results from the limited amount of sample material available. Each analysis required three days but was found to be satisfactory. Although there were no chromatograms published by Clamp et al. (1967) the order of elution of the trimethylsilyl derivatives of the carbohydrates were the same in this study. Good peak resolution was obtained using the linear temperature program of 4°/min from 80°C to 200°C, sialic acid was eluted after the final temperature had been reached though peak symmetry and width was still acceptable. No advantage would have been gained using the temperature program of Clamp et al. (0.5°/min, 140°-200°C), in fact the higher starting temperature was found to be a distinct disadvantage due to excessive tailing of the initial solvent and excess reagent peak. The methyl glycosides were prepared by hydrolysis in 0.75 NHCl in anhydrous methanol which eliminated the requirement of a separate method for the determination of N-acetyl neuraminic acid. Silylation was carried out using a mixture of pyridine, trimethylsilyl chlorsilane and hexamethylenedisilazane. All -OH groups are silylated giving highly volatile derivatives which were separated on a column using SE30 as the liquid phase. According to Wheelock and Sinkinson (1969) the re-N-acetylation step can be omitted, their shorter preparation time for preparation of the methyl glycosides (3 hours) was found to be insufficient to give complete hydrolysis. The 6-hour reflux time used in this study probably caused some hydrolysis of N-acetyl-galactosamine but re-N-acetylation according to Clamp et al. (1967) was not found to be reliable.

The Millipore Pellicon membrane filters are used in a variety of applications such as concentration of macromolecules and the recovery of macromolecular solutes, fractionation of macromolecules on the basis of molecular weight, purification and concentration of bacterial and viral cultures and in detoxification of biological products. In this study a method was required for the study of agglomeration of proteins and to separate any carbohydrates released from the casein fractions after enzyme treatment. Although slow filtration through the 100,000 filter was sometimes found, no problems were encountered with the 25,000 and 1,000 filters. The use of filters, in preference to dialysis, in the attempted recovery of carbohydrates released from the proteins, obviated the need for reducing large volumes of dialysate to dryness before hydroysis and derivatization. This reduced the risk of contamination and speeded up the analysis.

B. Discussion of Results

At the beginning of the experimental work on this project it was proposed to use rennin as a reference since its action on the caseins has been widely discussed in the literature. Gel electrophoresis results showed little proteolysis of calcium caseinate though considerable destabilization takes place giving rise to clot formation. The expected drastic effect on κ-casein was well illustrated (Fig. 6b, 7b), its marked effect on α_s-casein (Fig. 3b) and to a lesser extent on β-casein (Fig. 5b) was clear evidence of the general proteolytic properties of the enzyme. Although α_s-casein stays in solution after rennin treatment only a very small amount will pass the 100,000 filter (Fig. 11dS, 11eS1) which would indicate aggregation taking place in addition to the degradation by rennin. The slight proteolysis of β-casein by rennin is not accompanied by aggregation to the same degree as with α_s-casein. κ-casein after rennin treatment leaves nothing in solution detectable by gel electrophoresis, the precipitate shows highly aggregated para-κ-casein of which only a small amount passes the 100,000 filter after SDS treatment (Fig. 18eP, 18fP1). Strong inter-molecular disulphide bonding must be one of the main causes since the addition of DTT before electrophoresis allows migration of the para-κ-casein to the 12,000 dalton region on the gel. Although only minor proteolysis can be detected on the calcium caseinate gels after rennin treatment a definite reduction in the amount passing the 100,000 filter was seen (Fig. 21aP, 21bP1), as compared to the untreated sample, indicating aggregation taking place in this case. Trichloracetic acid soluble nitrogen endorsed the gel electrophoresis work, a considerable amount of soluble nitrogen was detected in both 12% and 2% TCA after rennin treatment of κ-casein; the amount soluble in 12% TCA due to the macroglycopeptide (Nitschmann and Beeby, 1960). Detectable amounts of soluble nitrogen in 2% TCA were found due to rennin treatment of α_s-casein and calcium caseinate.

The amount of soluble nitrogen in TCA after rennin treatment of calcium caseinate and κ-casein compares favorably with published data. On a percentage basis the final amount of soluble nitrogen after rennin treatment of calcium caseinate is 8% of the total original amount in 2% TCA; and 4% of the total is soluble in 12% TCA compared to 4% and 1.5%, respectively from whole casein (Nitschmann and Bohren, 1955). Approximately two-thirds of the nitrogen soluble in 2% TCA after rennin treatment of κ-casein is precipitated with 12% TCA, concurring with the results of Nitschmann and Beeby,(1960).

Carbohydrate analysis showed that rennin released the macroglycopeptide from κ-casein which was able to pass the 25,000 filter but not the 1,000 filter. Since the estimated molecular weight of the macroglycopeptide is 8,000 daltons the results are consistent with expectations. No carbohydrates could be found in α_s- and β-caseins after methanolic hydrolysis of the untreated fractions. If methanolic hydrolysis had failed to release bound carbohydrates, then after proteolysis with rennin, the carbohydrates may have been made more accessible; however, carbohydrate analysis failed to reveal any carbohydrates released from α_S- or β-casein due to rennin action.

Trypsin was only used in the initial studies and its action was nearly identical in every case with the production of a 9,000 dalton band. The staining intensity of this band would indicate that it does not account for all the original substrate, other fragments may have passed right through the gel or were so diffuse as to be incapable of visualization after staining. The 9,000 dalton band is quite discreet and may infer that all casein fractions contain a relatively small macropeptide resistant to proteolysis.

The gels of lysozyme treated caseins showed no evidence of degradation, however a small amount of α_S-casein was precipitated from solution. The majority, which stayed in solution, would not pass the 100,000 filter (Fig. 12dS) indicating considerably more aggregation than the untreated α_S-casein. Aggregation of the β-casein, α_S-casein and calcium caseinate was not detected compared to untreated samples and no TCA soluble nitrogen was found due to the action of lysozyme on any of the casein fractions, indicating no protein degradation. No carbohydrates were released from α_S-, β- or κ-caseins by lysozyme. The mode of action of lysozyme in α_S-casein aggregation is obscure, particularly since α_S-casein is effectively devoid of cystine or cysteine residues which are prominent in protein aggregation. The carbohydrates in κ-casein were not affected, but visual observation of the effect of lysozyme on calcium caseinate showed destabilization. This destabilization must be due, in part, to the aggregation of α_S-casein by lysozyme.

β-glucosidase showed a marked degradative effect on α_S-casein, particularly the more pure preparation (Fig. 2d, 3d). This breakdown of α_S-casein was not accompanied by aggregation, as the treated samples showed similar filtering characteristics compared to the untreated sample (Fig. 13). Some degradation of β-casein was detected due to β-glucosidase (Fig. 5d) but, once again, no aggregation (Fig. 17). Limited degradation of κ-casein was evident due to β-glucosidase, with the soluble fraction having the same molecular weight as para-κ-casein (Fig. 6e, 7e). Neither the precipitate nor the soluble fraction were filterable through the 100,000 filter, indicating aggregation (Fig. 19eP, 19fS). This was in contrast to untreated κ-casein in the case of the precipitate. The effect of β-glucosidase on calcium caseinate was not clear, aggregation took place (Fig. 9d) and the bands in the gels became blurred (Fig. 23). This phenomenon may be due to the transglycosylation reaction catalyzed by the enzyme. Although this activity is not the major one it has been shown that β-galactosidase will form oligosaccharides from lactose (Wierzbicki and Kosikowski, 1973). Lactose was surprisingly hard to remove from calcium caseinate; Wheelock and Sinkinson (1971) found that lactose remained in micelle preparations even after dialyzing against running water for several days. With high bound or associated carbohydrate content proteins are difficult to electrophorese under the conditions used in this study and blurring of bands might be expected.

The TCA soluble nitrogen analysis of β-glucosidase treated caseins endorsed the results of the gel electrophoresis. In 2% TCA there were appreciable amounts soluble from the α_S- and κ-casein samples and small amounts from β-casein and calcium caseinate. No major amounts were found soluble in 12% TCA, the soluble peptide would therefore not be expected to contain carbohydrates. The carbohydrate analysis of β-glucosidase treated caseins did not reveal any removal of carbohydrates from the proteins, the trisaccharide of κ-casein did not appear to be affected. β-glucosidase did not appear to be promoting the removal of carbohydrates from the proteins; it did however, cause aggregation of κ-casein and promote degradation of α_s-, β- and κ-casein. The cumulative effects of β-glucosidase would enable destabilization of the micelle though the specific action of this enzyme on calcium caseinate and the purified fractions could not be defined in this study.

The visual observations of the casein fractions treated with rennin, trypsin, lysozyme and β-glucosidase clearly indicated that the enzymes were affecting calcium caseinate and κ-casein; in addition lysozyme showed some effect on α_s-casein. As no evidence of protease contamination of the glycolytic enzymes could be found the visual effects must be the result of the properties of the glycolytic enzymes. The effects of rennin have been studied extensively (Mackinlay and Wake, 1971), although this study did reveal some interesting new data concerning its effects on the caseins, particularly in regard to the aggregation phenomena. The studies of Bakri and Wolfe (1971) and Wolfe (1971) had shown that lysozyme and β-glucosidase would destabilize the milk micelle. Bakri and Wolfe, working on a micelle preparation, assumed that the carbohydrate moiety of κ-casein was being affected in some way by lysozyme though this study did not produce any evidence to substantiate this theory. In the work of Wolfe curd formation due to the action of β-glucosidase on whole milk was studied. No other data on the effects of lysozyme and β-glucosidase on the caseins has been published.

Several researchers have pointed out that it is difficult to relate the effects of enzymes on purified casein fractions to the effect on the micelle. The extraction and purification of the fractions requires the use of large quantities of chemicals which may denature the proteins. The secondary and tertiary structure of the proteins would be altered during purification and it is doubtful if the proteins would return to their normal state after the removal of these chemicals. The stabilizing power of κ-casein against α_s-casein precipitation with calcium chloride varies according to the method of κ-casein purification (Zittle and Custer, 1963). The use of ethanol to precipitate the protein sometimes caused a reduction in the stabilizing power of κ-casein and was thought to be due to denaturation. Purified fractions are stripped of the stabilizing effects of each other and the contribution of inorganic ions; therefore they may behave quite differently under these conditions. In this study this point is well illustrated as the purer preparations showed much more susceptibility to enzyme action than the less pure proteins. Studies on the pure casein fractions can pinpoint the properties of the proteins and their susceptibilities to different treatments; these features may not be as obvious when studying the intact micelle or may be masked. A case in point is the degradation of κ-casein by rennin, this can be better illustrated using purified preparations of κ-casein.

Rennin has been shown to initially attack a phenyl-alanine methionine bond in κ-casein (Delfour et al., 1965) and release a peptide containing the trisaccharide moiety. In the theories of micelle destabilization great emphasis has been put on this point. Although the carbohydrates certainly contribute to the hydrophilicity of the protein the κ-casein itself is more hydrophilic than either α_s- or β-casein and any loss of κ-casein as a whole or as fragments would cause destabilization.

Rennin, lysozyme and β-glucosidase all have the common property of affecting α_s-casein. In this study it was found that with α_s-casein rennin caused degradation and aggregation, lysozyme caused minor precipitation but major aggregation and β-glucosidase caused degradation without aggregation. These phenomena were demonstrated in the absence of calcium which is known to cause precipitation of α_s-casein. It is apparent from the results of this study that precipitation cannot be taken as the sole criterion of enzyme induced modification of α_s-casein or of other caseins. The filtration results indicate that aggregation does not have to be accompanied by degradation, in the case of lysozyme the effect is subtle and should warrant further study of the effects of this enzyme on proteins, particularly with respect to possible catalysis of side group modifications.

α_s-casein is heterogeneous but the major portion is known, in more strict casein protein nomenclature, as α_{s1}-casein and it is this fraction that exhibits the genetic variants which can be separated by starch gel electrophoresis (Thompson et al., 1962). These are termed α_{s1}-A, α_{s1}-B, α_{s1}-C and α_{s1}-D caseins. These genetic variants do not differ very greatly in molecular weight, solubility or phosphorus content and have identical terminal end groups. The slight differences in amino acid sequences and contents are enough to give different mobilities and separation by electrophoresis.

In the SDS gel electrophoresis results lysozyme was shown to precipitate a minor portion of α_s-casein (Fig. 12), rennin and β-glucosidase treatment showed degradation of α_s-casein (Fig. 11, 13). The minor effect of lysozyme precipitation may be due to its action on minor protein fractions which are known to be associated with α_{s1}-casein and precipitated with α_{s1}-casein in the purification procedures used in this study. These minor associated α_s-caseins differ considerably from α_{s1}-casein.

Annan and Manson (1969) prepared fractions of α_s-casein which were not genetic variants. α_s-casein was subjected to starch gel electrophoresis at pH 9.2 giving six clearly defined bands which were coded according to mobility. α_{s0}-casein ran ahead of the major component α_{s1}-casein; the others, α_{s2}-, α_{s3}-, α_{s4}- and α_{s5}-casein, coded in order of decreasing mobility. α_{s5}-casein has since been tentatively identified as a dimer of α_{s3}- and α_{s4}-casein, probably linked together by a disulphide bond. The molecular weight of α_{s5}-casein has been calculated to be 65,750 daltons by Toma and Nakai (1973) and 67,500 daltons by Hoagland et al. (1971). α_{s3}- and α_{s4}-caseins have identical molecular weights at 31,800 daltons reported by Toma and Nakai (1973) or 33,700 daltons reported by Hoagland et al. (1971). Although these minor α_s-caseins qualify as calcium sensitive, stabilized by κ-casein and precipitate with α_{s1}-casein there are some significant differences. The most notable difference is the presence of cystine or cysteine in the α_{s5}-casein, these residues are not present in α_{s1}-casein. κ-casein does not interact with α_{s5}-casein to the same extent as with α_{s1}-casein to prevent calcium precipitation. These minor α_s-caseins are ignored in micelle structure theory as indeed are the whey proteins. It would be interesting to determine the effect of rennin, lysozyme and β-glucosidase on these purified α_s-casein fractions, as these enzymes are known to affect crude α_{s1}-casein.

α_s- and β-caseins apparently contain little ordered secondary and tertiary structure. This is not surprising as the proline content of s1-casein and β-casein is high, 20 residues/mole and 33 residues/mole, respectively (Mercier et al., 1971). This amino acid is known to interrupt the helical and β-structures in proteins.

The charge and average hydrophobicity of α_s- and β-caseins varies considerably in different segments of the proteins (Farrel, 1973). The N-terminal portion of β-casein is the least hydrophobic, the major portion of the net negative charge of the protein found in the first 43 residues from the N-terminal end. α_s-casein contains seven of the eight phosphate residues and twelve carboxyl residues between residues 43 to 80 of the protein and is the least hydrophobic portion.

Only minor changes could be detected in the gel patterns of enzyme treated calcium caseinate, which would indicate that only limited modifications of the constituent proteins were responsible for destabilization or clotting. The factors responsible for keeping the micelle in colloidal suspension are in delicate balance and proteolysis is not the sole cause of destabilization. The caseins are some of the most hydrophobic proteins known (Bigelow, 1967) and small changes in α_s-casein structure could have a profound effect upon the integrity of the micelle. In the theories of micelle structure it is proposed that there is an intimate relationship between α_s- and κ-casein and between β- and κ-caseins. In the case of the action of rennin and β-glucosidase, the degradation of α_s- and β-casein may upset the association of κ-casein with α_s- and β-casein and any loss of κ-casein associated with the degradation would have a destabilizing effect on the micelle. From the results of this study it would seem that modifications of α_s- and β-casein were important in the destabilization of the micelle.

The aggregation of the proteins in calcium caseinate by β-glucosidase is not accompanied by any visual evidence of α_s-casein degradation nor by any marked increase in the amount of TCA soluble peptides. There is no visual evidence of α_s- or β-casein degradation due to rennin treatment in the calcium caseinate gels. The micelle is thought to have a porous structure (Ribadeau-Dumas and Garnier, 1970), which can be penetrated by rennin and by other enzymes. If enzymes could penetrate the micelle there should be evidence of protein degradation. It would appear from the evidence from this study, that κ-casein may be predominantly situated on the outer layers of the micelle and enzymes do not penetrate the micelle itself. Rennin and β-glucosidase both degrade purified α_s-casein and both enzymes should be small enough to penetrate the micelle (on the evidence of Ribadeau-Dumas and Garnier) but no loss of α_s-casein band intensity is seen in the calcium caseinate gels. Therefore, the degradation must be either inhibited or the enzymes are unable to penetrate the micelle. The basis for the theory that the micelle is porous, according to Ribadeau-Dumas and Garnier, is the fact that carboxypeptidase A quantitatively removes C-terminal residues from α_s-, β- and κ-caseins. However, in the micelle model of Waugh et al. (1970) the rosette formation of α_s- and β-casein envisages the N-terminal protions of the proteins to be highly associated at the center and the C-terminal portions to the circumference. This configuration would allow carboxypeptidase A to attack the C-terminal residues without having to penetrate to the center of the submicellar particle. κ-casein would also be accessible since the molecule is thought to be associated with the C-terminal portions of α_s- and β-casein. By the calculated size of the degradation products of rennin and β-glucosidase action on the casein fractions, the peptide linkages which are attacked must be some distance from the C-terminal end of the proteins and indeed could be closer to the

N-terminal end. The closer to the N-terminal region of α_s- and β-casein that cleavage occurs the more difficult it would be for the enzymes to penetrate which may result in the lack of α_s-casein degradation in calcium caseinate.

The theory that the carbohydrate moiety of κ-casein could be modified or removed by lysozyme or β-glucosidase in order to destabilize the micelle was disproved in this study. The action of these two enzymes remains obscure, the effects were demonstrated but the mechanisms of destabilization were not found. Rennin, lysozyme and β-glucosidase do not have drastic degradative effects on calcium caseinate and yet are able to induce destabilization. In the light of the findings of this study the trisaccharide of κ-casein is not of paramount importance in the stability of the micelle. No evidence was gained to support a theory that any one of the constituent proteins was more important than another in micelle stability. It was not found possible to relate the effects of the enzymes on the casein fractions to the effect on the micelle. It is quite possible that lactose, which was found difficult to remove from calcium caseinate, and whey proteins, which must be able to penetrate the micelle according to the theory of the porous nature of the micelle, may contribute more to the stability of the micelle than is presently thought.

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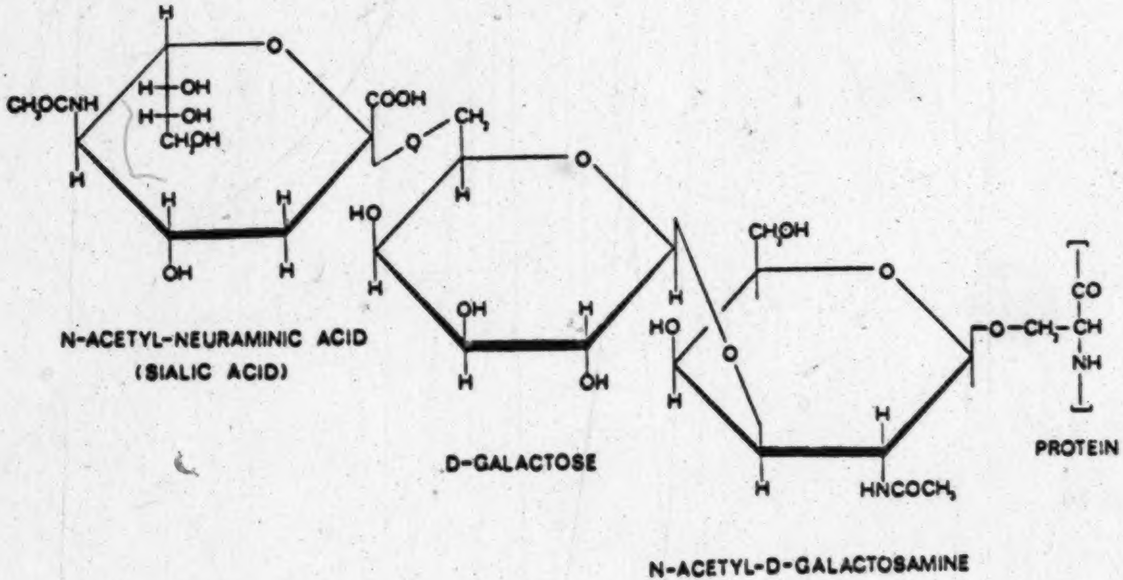
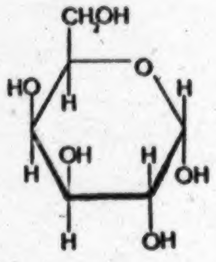
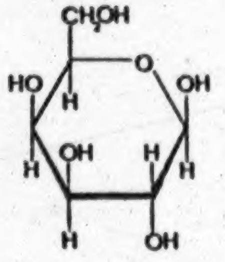


APPENDIX I. Probable structure of trisaccharide in κ-casein (Tran and Baker, 1970).



α-D-galactopyranose



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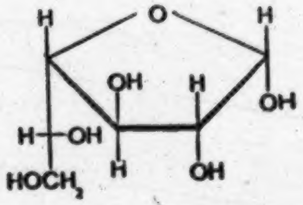
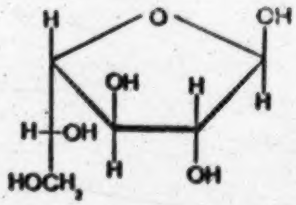
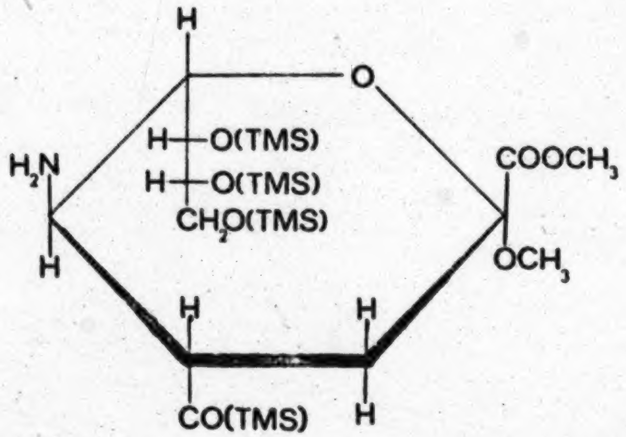


α-D-galactofuranose

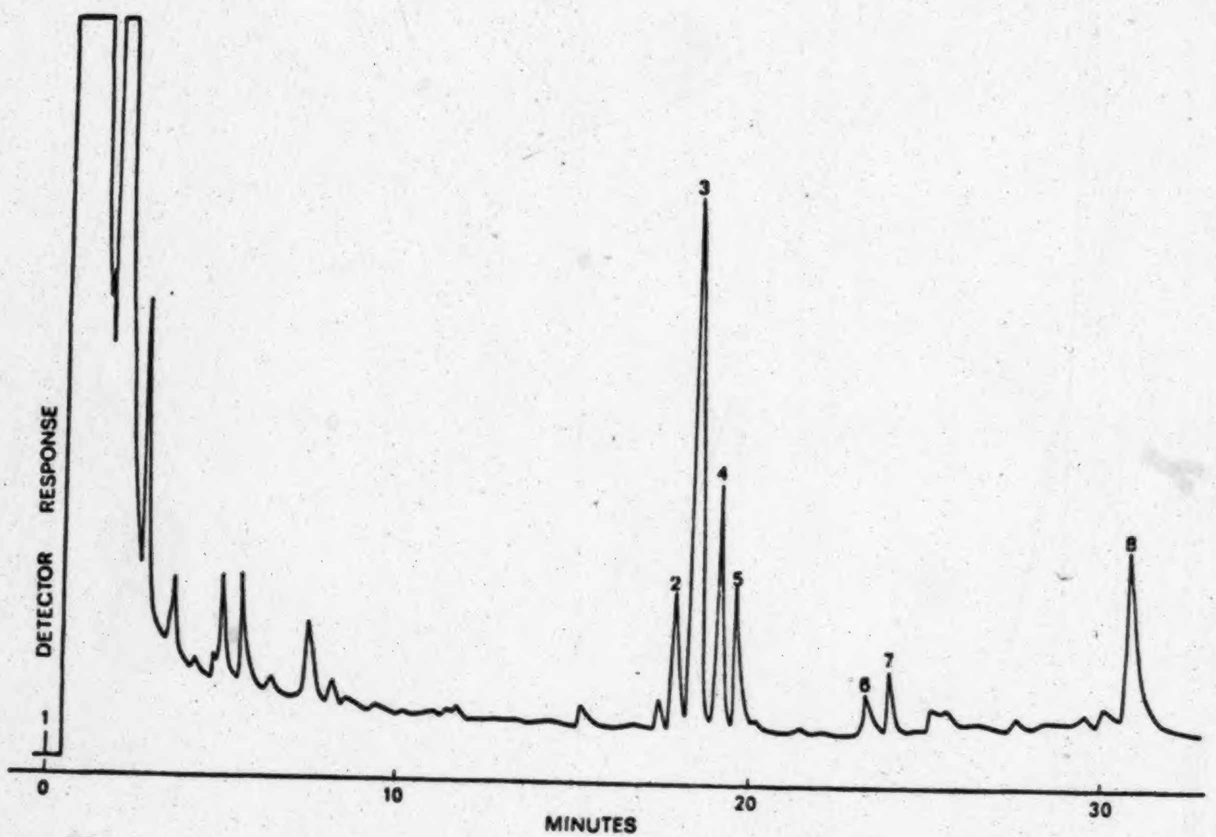


β-D-galactofuranose

APPENDIX II. Haworth formulas for the ring structures of D-galactose



APPENDIX III. Trimethylsilyl derivative of sialic acid



APPENDIX IV. Typical chromatogram of trimethylsilyl carbohydrate derivatives from κ-casein. 1, injection point; 2, γ-D-galactose; 3, α-D-galactose; 4, β-D-galactose; 5, D-galactosamine; 6 and 7, N-acetyl-D-galactose; 8, sialic acid. Conditions: hydrogen 30 ml/min, air 250 ml/min, nitrogen 25 ml/min; glass column 6' x 2 mm i.d.; support Chromosorb W, High Performance; liquid phase 3% SE 30; injector and detector 250°C; temperature program 4°/min 80° to 200°C.

APPENDIX V. Description of enzymes used in this study

Rennin (3·4·4·3), or as it is sometimes called chymosin, is described as peptidyl-peptide hydrolase. Its activity is standardized by its milk clotting ability; one rennin unit is defined as the rennin activity which will clot 10 ml of reconstituted milk substrate in 100 seconds at 30°C (Berridge, 1945). Since the substrate is subject to variations, the British Standards Institution supplies ampules of freeze dried rennet of known activity for standardization.

Trypsin (3·4·4·4) is also described as a peptidyl-peptide hydrolase. Extracted from beef or hog pancreas the enzyme is assayed using N-benzoyl-L-argenine ethyl ester, p-toluene-sulfonyl-L-arginine methyl ester or casein as the substrates, the units of activity are related to the substrate employed. The enzyme hydrolyzes peptides, amides and esters at bonds involving the carboxyl groups of L-arginine and L-lysine.

Lysozyme (3·2·1·17) is an enzyme which hydrolyzes mucopolysaccharides present in the cell walls of certain bacteria and slowly hydrolyzes chitin. It is found in tears, nasal mucus, saliva and blood serum of humans and in a number of tissues and secretions of different animal vertibrates and invertebrates and in plants. The activity of lysozyme is assayed by observing the rate of lysis of _Micrococcus lysodeikticus_ by spectrophotometric measurements at 540 mμ. One unit of activity corresponds to an increase in optical density of 0.001 per minute at pH 7 and 25°C. Lysozyme occurs in bovine milk at the rate of 13 μg/100 ml in human milk it is present at 40,000 μg/100 ml (Chandan _et al._, 1968) and is thought to contribute to the antibacterial activity in human milk. The lysozymes of different sources vary in chemical composition and in affinities for _Micrococcus lysodeikticus_.

β-glucosidase (3·2·1·21) extracted from almonds is described systematically as a β-D-glucoside glucohydrolase. It is a common plant enzyme which catalyzes transglycosylation or more commonly hydrolysis reactions with β-D-glucosides. The assay of β-glucosidase is carried out by following the hydrolysis of salacin, one unit of activity corresponds to the liberation of 1 μmole of β-D-glucose per minute at 37°C (Nelson, 1944).